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The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 44 SEPTEMBER, 1954 NO. 3

The Official Publication of the Central Society for Clinical Research

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Vol. 44, No. 3, September, 1954. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription price: United States and its Possessions $12.00 per year; Canada $12.50; Foreign $13.50. Subscriptions to medical students, interns and residents, half-price. Entered as Second Class Matter October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

The Journal of Laboratory and Clinical Medicine

VOL. 44 SEPTEMBER, 1954 No. 3

CLINICAL AND EXPERIMENTAL

INFECTIOUS ECTROMELIA OF MICE (MOUSE POX)

RICHARD E. SHOPE,* M.D., NEW YORK, N. Y.

DURING the fall of 1952, members of the Virus and Rickettsial Study Section of the Division of Research Grants, National Institutes of Health, heard rumors that infectious ectromelia was present in certain mouse colonies of the United States. With the knowledge that the presence of such an infectious agent constituted a threat to the progress of medical and other research, the Study Section named a committee consisting of Richard E. Shope, Chairman, Karl F. Meyer, Frank L. Horsfall, Jr., W. T. S. Thorp, and Edward P. Offutt, Executive Secretary, to consider this situation and the problems it presented, and to make recommendations to the proper authorities.

The Committee's first activity was to advise research grantees of the situation by individual notices sent to approximately 2,000 individuals widely scattered over the United States and in a few foreign countries. It requested cooperation of all grantees in reporting suspected outbreaks and established infections. To date, somewhat over 1,200 replies have been received, with a breakdown in the following simple fashion:

No mouse stocks present	746
No unexplained disease present	440
Unexplained disease (low mortality makes ectromelia less likely)	13
Infectious ectromelia believed present	18
Infectious ectromelia proved present	4

With reference to the suspected ectromelia cases, infection has not been confirmed in any instance so far as is known. At the present time in these colonies disease is apparently not a problem. Three of the four proved cases have been reported.[10-12]

This report has also been submitted to the following journals: Journal of Bacteriology, American Journal of Tropical Medicine and Hygiene, Journal of the American Veterinary Medical Association, Journal of the National Cancer Institute, and Science.

Received for publication, June 10, 1954.

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Besides presenting the afore-mentioned results of its survey, the Committee wishes more widely to advise research users of the mouse, and suppliers of this animal, of the following basic facts concerning ectromelia, or mouse pox.[2, 5, 7, 9]

1. *Nature of Disease.*—

A virus infection, first described by Marchal at Hampstead (1930). Virus: 100-150 mμ in diameter, widely spread throughout body of animal, demonstrable in liver and cutaneous tissue, destroyed by 55° C. for thirty minutes, withstands 50 per cent glycerol in the refrigerator for several months, withstands 0.5 per cent phenol, confers rather substantial immunity in recovered animals, related to vaccinia and other animal pox viruses.

Highly infectious, often epizootic.[6] All known strains of mice susceptible, some more so than others (as A, BC, greater than C 57) with generally a high mortality. For a study of its epidemic spread, see Greenwood Hill, Topley, and Wilson.[8]

2. *Recognition.*—

Acute disease: characterized by visceral lesions; animals may die without apparent cause, appearing in good health until a few hours or a day before death, dying without readily apparent external lesions. One death usually followed by deaths of other contacts within a few days to a few weeks. Usually spreads throughout colony in six to eight weeks with a 50 to 95 per cent mortality. Inconspicuous primary lesions sometimes present. In other instances, swollen eyelids, swollen pocked nares and pock-like lesions on body may be prominent signs.

First evidence: ruffled hair, puffy appearance of face, i.e., nonspecific.

Gross autopsy findings: in fulminating case none of these findings may be present, or only some may be: (a) pale, dirty grey or putty-colored liver, or red liver mottled with white areas of focal necrosis; (b) slightly enlarged spleen, sometimes, in long-standing cases, with large white areas; (c) hemorrhagic appearance of proximal inch or two of small intestine; (d) sometimes excess fluid in peritoneal cavity and sticky, stringy exudate covering intestines; (e) pulmonary congestion or consolidation, sometimes with excess pleural fluid.

Microscopic anatomic findings:

(a) necrosis of mesoblastic tissues.

(b) large eosinophilic (acidophilic) intracytoplasmic inclusion bodies in epithelial cells of skin and epithelium of intestine (not always present in fulminating disease).

Chronic disease: characterized by necrosis of extremities. Usually one foot swollen and edematous, serous fluid escapes from surface forming minute scabs: (a) progresses to gangrene and sloughing of member, leaving healthy tissue, or, (b) spreads to another foot, to tail or mouth, perhaps eventually causing death.

Latent symptomless form: almost impossible of recognition. *Reservoir* of virus. The chronic disease is characterized by colony infection. The disease is transmitted from mother to offspring to be manifest irregularly as described previously under chronic disease.

3. *Differentiation.*—

Chronic foot lesions must be distinguished from those caused by *Streptobacillus moniliformis*; this organism may be cultivated on serum agar, Loeffler's medium under microaerophilic conditions, or in equal parts of serum and broth.

4. *Diagnosis* (See also Reference 4).—

(a) *Demonstration of virus:* One method: grind portion of suspect liver and spleen in small amount of saline solution. Inoculate intraperitoneally into normal mice. If virus is present, animals will usually die in four to six days with macroscopically visible liver lesions.

(b) *Serologic diagnosis:* Sera of mice recovered from ectromelia inhibit red cell agglutination by vaccinia virus.[3]

5. *Treatment.*—
None known.

6. *Prevention.*—
(a) Strict isolation from outside contamination. This also includes adequate facilities for handling mouse colony and *noncirculating system if air conditioning practiced.*
(b) Possible that vaccination of individual mice by scarification method or intranasal instillation using vaccinia virus or formalinized liver suspension from infected mice (Greenwood and Topley, 1936) may be of benefit.[1] Probably impractical in most cases except for particularly valuable inbred stocks.

7. *Control.*—
(a) Prompt recognition of infection;
(b) Extermination of infected colonies by destruction and incineration of infected animals and contacts.
(c) Sterilization of quarters.

8. *Recommendations.*—
(a) Extermination of known infected colonies.
(b) Destruction of materials harvested from infected colonies.
(c) Isolation and self-imposed quarantine of colonies suspected of infection.
(d) Prompt reporting of all suspected and proved infections.
(e) Restriction of shipments of mice between investigators.
(f) Treat all materials such as blood, serum, tissues, or ectoparasites harvested from mice, particularly from *foreign* sources, as potentially contaminated and handle with all precautions necessary.
(g) Familiarizing of scientists with the existence of legislation prohibiting the importation or interstate movement of ectromelia virus or animals or materials potentially infected with it.

(An Act approved Feb. 2, 1903 [32 Stat. 792; 21 U.S.C. 111] and BAI Order 381 [9 Code of Federal Regulations, Parts 101-123], regarding the importation or interstate transportation of viruses or vectors [animals inoculated or exposed thereto] have been interpreted as applicable to infectious ectromelia. A permit from the United States Department of Agriculture is, therefore, required for the importation or interstate movement of ectromelia virus or materials infected with the virus.)

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STUDIES ON THROMBOPLASTIN GENERATION

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ANIMAL tissues are known to contain a potent thromboplastin, which, under certain conditions, rapidly converts prothrombin to thrombin. Furthermore, it is generally agreed that the precursors of a substance similar in action are present in the circulating blood.[1] In some manner, disruption of vascular continuity seems to initiate their combination or release to produce a spontaneous thromboplastin. No agreement has been reached, however, on the identity, nature, and mode of action of the precursors.

Platelets were thought originally to contain a preformed thromboplastin that was released when they come into contact with disrupted vascular surfaces.[2] More recent evidence has suggested that there are required precursors in both platelets and plasma. Brinkhous[3] postulated a "thrombocytolysin" which facilitates release of thromboplastin from the platelets. Quick[4] suggested that platelets release an enzyme that activates a plasma "thromboplastinogen" to yield thromboplastin. Shinowara[5] found that the combination of a blood cell derivative with a plasma derivative in the presence of calcium produces a thromboplastin comparable in strength to placental thromboplastin. Finally, Conley and associates[6] has suggested that precursors of thromboplastin are present in plasma alone, since "platelet-free" plasma was found to clot after contact with glass surfaces.

Tocantins[7] has viewed the fluidity of blood as representing a balance between forces promoting coagulation and those inhibiting it. In this hypothesis, clotting results when the promoting forces are more active than the anticoagulants; thus he believed that disrupted vascular surfaces either adsorb or inactivate antithromboplastin and, in addition, furnish thromboplastin so that the latter and platelet thromboplastin are free to initiate clotting. He found that antithromboplastin was increased at least several fold over normal in hemophilia, and believes this to be the primary defect in the disease.[8]

Most workers, however, consider hemophilia to result from deficiency of a plasma thromboplastin precursor. Patek and Taylor[9] found a substance present in normal human plasma, but absent or inactive in hemophilic plasma, which corrected the clotting time of hemophilic blood. This antihemophilic globulin[10] has generally been equated with thrombocytolysin,[3] thromboplastinogen,[4] thromboplastin plasma component,[5] and the plasma precursor of Conley and associates,[6] since each of these was considered deficient in hemophilia by the respective investigators.

It is apparent from this brief introduction that, in view of well-founded differences of opinion, re-evaluation of the first stage of coagulation is needed. The need becomes even more apparent with the recent reports of Christmas disease[11] and similar disorders,[12-14] and of plasma thromboplastin antecedent (PTA) deficiency,[15] since in these diseases there is defective thromboplastin formation and the deficient factors are not antihemophilic globulin.

The thromboplastin generation test presented by Biggs and associates[16, 17] offers an excellent means to investigate the first stage of coagulation. It was decided to use comparable techniques to study thromboplastin generation, especially with regard to the differences of opinion mentioned previously and to the role of the Christmas factor[11] (plasma thromboplastin component[14]). It is the purpose of this paper to report some of our findings.

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Received for publication June 17, 1954.

MATERIALS AND METHODS

Platelet-Poor Plasma.—Unless otherwise indicated, all glassware and plastic with which platelets came into contact prior to final use had been coated with General Electric Drifilm #SC-87, rinsed twenty or more times with distilled water, and dried. Needles and syringes were coated with silicone oil. Normal human blood, obtained by carefully performed venipuncture using the two-syringe technique, was added to 0.1 M sodium oxalate in a plastic tube, nine parts of blood to one of oxalate. The latter was then capped with Parafilm, gently inverted twice, and centrifuged in an angle centrifuge for ten minutes at 1,500 r.p.m. Most of the platelet-rich plasma was transferred to a smaller plastic tube and centrifuged at 4,000 r.p.m. for fifteen minutes; the clear supernatant plasma was then separated from the platelet button. Plasma to be used the same day was refrigerated at 5° C.; otherwise it was frozen.

"Whole Platelets."—The platelet button was washed with 2 ml. of 0.85 per cent sodium chloride, resuspended by shaking, and centrifuged at 4,000 r.p.m. for fifteen minutes. This step was repeated, and the final button was suspended in an amount of saline equal to one-third of the original volume of platelet-rich plasma. Best results were obtained when platelets were handled in a cold room at 5° C.

Triturated Platelets.—These were prepared just as whole platelets, except that with each washing the platelets were transferred to a glass homogenizer and triturated for several minutes. The final button of platelet particles was then frozen. For use it was thawed and again triturated in an amount of saline equal to one-third of the original volume of platelet-rich plasma. Frozen platelet particles seemed to be comparable in activity to fresh platelets and could be kept for many days.

Plasma Thromboplastin Component (PTC),[14] *(Christmas Factor*[11]*).*—PTC was obtained from normal human serum by adsorption on barium sulfate, subsequent elution by sodium citrate, and pH adjustment to destroy SPCA and prothrombin.[14] The preparation was dialyzed against 0.85 per cent saline, adjusted to the original serum volume, and frozen in 0.5 ml. portions. No other known coagulation factors have been detected in this preparation. When the serum from a patient with PTC deficiency was used as a source of PTC, a preparation resulted that was inert when used for thromboplastin generation.

Antihemophilic Globulin (AHG).—AHG was prepared from normal human barium sulfate adsorbed plasma by one-third saturation with ammonium sulfate as described by Biggs, Douglas, and Macfarlane.[17] The final product was adjusted to the original plasma volume and kept frozen in 0.5 ml. portions until used. This preparation corrected the delayed recalcification time and poor prothrombin consumption of hemophilic plasma. When plasma from a patient with hemophilia was used as a source of AHG, a preparation resulted which was inert when used for thromboplastin generation.

Serum.—Normal human blood was placed in a glass tube and allowed to clot. As the blood clotted, the tube was agitated on a slow rotor and rimmed twice with an applicator stick to obtain maximal prothrombin consumption. After one hour the serum was separated and incubated for twenty minutes at 37° C. to allow thrombin destruction. In every case the serum prothrombin time was greater than sixty seconds.

Calcium Chloride.—Calcium chloride, 0.025 M, was used.

Imidazole Buffer.[18]—A stock solution was prepared as follows: 1.72 Gm. of Eastman Imidazole were added to 90 ml. of 0.1N HCl. To this was added 100 ml. of distilled water. For use the stock solution was diluted ten times with 0.85 per cent saline (pH 7.25). The presence or absence of this buffer made no apparent difference in the results.

A basic reaction mixture was prepared by mixing PTC, AHG, triturated platelets, and calcium chloride in equal parts. These ingredients were added in the order listed to a clean 13 by 100 mm. glass tube in a 37° C. water bath, and a timer was started upon the addition of calcium chloride.

Just prior to preparation of the reaction mixture, 0.1 ml. portions of platelet-poor plasma were pipetted with a 0.1 ml. pipette into 13 by 100 mm. glass tubes and kept in a refrigerator at 5° C. One minute before use, a tube of plasma was removed from the refrigerator and placed in the 37° C. water bath. At various time intervals, 0.1 ml. of the reaction mixture and 0.1 ml. of calcium chloride were added to 0.1 ml. of oxalated platelet-poor plasma and the clotting time measured with a stopclock. Thus, the mixture was used as a source of thromboplastin in a one-stage prothrombin determination.[1]

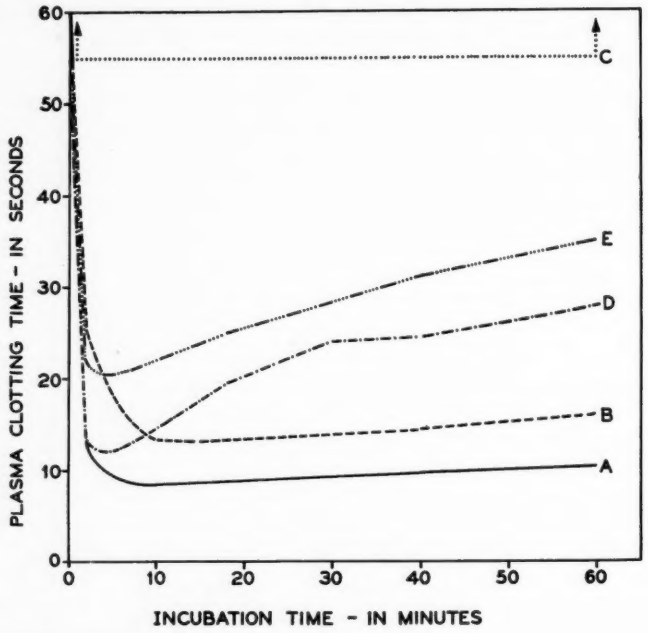

Fig. 1.—Activity of spontaneous thromboplastins; *A*, basic reaction mixture; *B*, basic reaction mixture without AHG; *C*, basic reaction mixture with saline replacing platelets, PTC, or calcium; *D*, basic reaction mixture with serum replacing PTC; *E*, basic reaction mixture without AHG and with serum replacing PTC.

To eliminate variation in reaction mixtures, measurements of two effects for comparison were made in the following manner: all basic ingredients except calcium chloride were combined and the mixture was halved and frozen in two tubes. Just prior to use, a tube was thawed and a timer started when calcium chloride was added. Activity was measured in the usual manner. All experiments were performed at least twice and most were carried out many times with similar results.

RESULTS

Basic Requirements for Thromboplastin Generation.—Powerful thromboplastin was generated by the basic ingredients. Within four minutes, it was sufficiently active to clot platelet-poor plasma in less than ten seconds (Fig. 1, *A*). That no appreciable amount of thrombin was present in the mixture is indicated by the fact that more than sixty seconds were required for it to clot a fibrinogen solution.

No thromboplastin was generated in the absence of PTC, platelets, or calcium (Fig. 1, *C*). When one of these was replaced by saline solution in the reaction mixture and the latter was added to plasma, fifty-five seconds or more were required to produce clotting. This slight activity was probably caused by the generation of thromboplastin *after* addition of the reaction mixture to plasma. In other words, it represents a "recalcification time."* The generation of thromboplastin in the absence of AHG is shown in Fig. 1, *B*. Although a relatively active thromboplastin is obtained, the yield is considerably less, and a longer time is required for its production.

Thromboplastin generated with the basic reaction mixture has the same activity when tested with hemophilic or PTC-deficient plasma as with normal platelet-poor plasma.

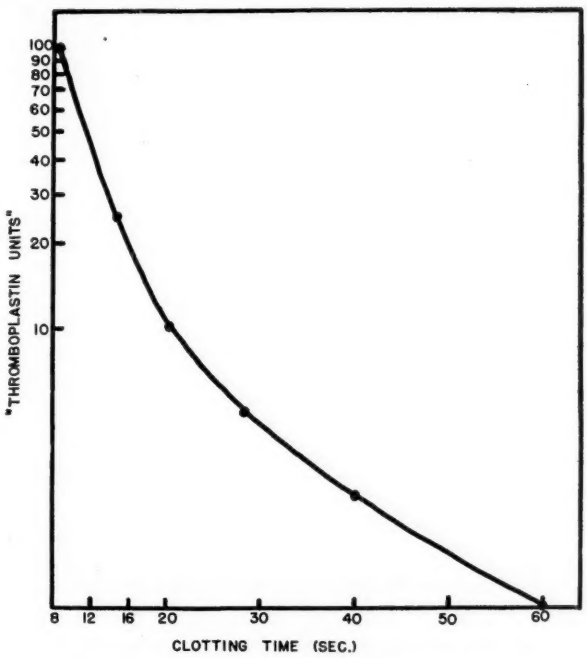

Fig. 2.—Thromboplastin dilution curve.

From these observations it is concluded that PTC, platelets, and calcium are necessary and probably sufficient for the generation of thromboplastic activity. AHG somehow increases the yield and rapidity of formation.

Quantitation of Thromboplastin.—For quantitation in further experiments, the "thromboplastin unit" was established by preparing multiple dilutions in saline solution of the basic reaction mixture at maximum activity, and measuring the time with which each dilution clotted platelet-poor plasma. Undiluted reaction mixtures usually reach a maximum activity of 8.3 seconds. When diluted one hundred-fold, such a mixture gives a sixty-second clotting time.

*0.125 ml. calcium chloride was added to a mixture of 0.1 ml. platelet-poor plasma, 0.025 ml. AHG, 0.025 ml. triturated platelets, and 0.025 ml. saline. Clotting occurred in 55 to 65 seconds.

Therefore, if the undiluted mixture is said to contain 100 thromboplastin units (per 0.1 ml.), then a mixture that gives a sixty-second clotting time contains one thromboplastin unit. A comparison of clotting time with thromboplastin units is given in Fig. 2.

Reaction Sequence.—Since at least three factors (platelets, AHG, and PTC) plus calcium are required for maximum thromboplastin production, the possibility that the reaction occurs in two stages is suggested. The following experiment was designed to check this possibility. Platelets, PTC, and calcium were incubated together for five minutes. Then one-half of the mixture was removed, added to AHG, and the thromboplastic activity of both mixtures followed. At the same time, a basic reaction mixture of PTC, platelets, AHG, and calcium was prepared and followed for activity. Fig. 3 shows the results of this experiment. By the preliminary incubation of PTC, platelets, and calcium, and the addition of AHG at zero time, the lag phase is eliminated and an activity curve (*A*) with a slope equal to the maximum slope of the basic reaction mixture (curve *B*) results immediately. (Difficulty in duplicating these results was encountered when greater platelet concentrations were used.)

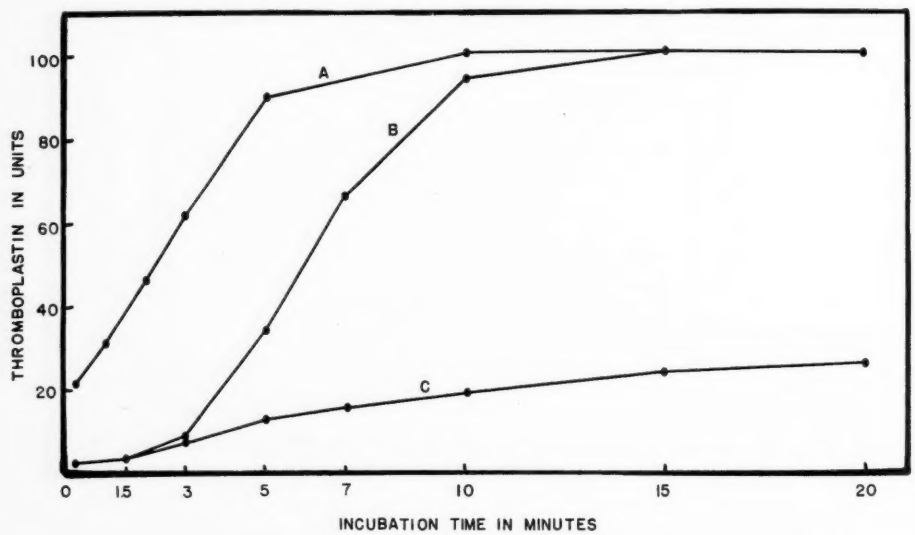

Fig. 3.—Elimination of lag phase, *A*, curve obtained upon addition of AHG to a mixture of PTC, platelets, and calcium after 5-min. incubation period; *B*, curve obtained at the same time as *A* with a basic reaction mixture; *C*, curve obtained from the incubation of PTC, platelets, and calcium.

No such elimination of the lag phase occurred when mixtures of AHG, platelets, and calcium, or of AHG, PTC, and calcium were incubated prior to the addition of the omitted factor. Incidentally, when plotted in thromboplastin units, the magnitude of the effect of AHG in the basic reaction mixture becomes much more apparent (compare curves *B* and *C*).

It is concluded, therefore, that PTC and platelets probably react together in the presence of calcium to form a weak thromboplastin and that a second reaction occurs in which AHG reacts rapidly in some manner with the thromboplastin to increase its activity.

Kinetics of the Reaction.—Various concentrations of the basic ingredients were used in order to learn more about the type of reaction.

1. Platelets. Fig. 4 illustrates the effect of varying triturated platelet concentration while keeping constant the concentrations of PTC, AHG, and calcium. This experiment was performed many times, and, in most cases, the final yield of thromboplastin was directly proportional to the concentration of platelets. With the two highest concentrations used the initial reaction velocity was the same, though this behavior was not apparent in the lower concentrations. These observations are inconclusive but suggest that platelets show the characteristics of a substrate.[19]

2. PTC. Fig. 5 illustrates the effect of varying PTC concentration while keeping constant the concentrations of platelets, AHG, and calcium. With all concentrations used the reaction rate was proportional to the concentration of PTC. Thromboplastin production with 50 per cent concentration of AHG approaches that of 100 per cent, though a longer time is required. This behavior

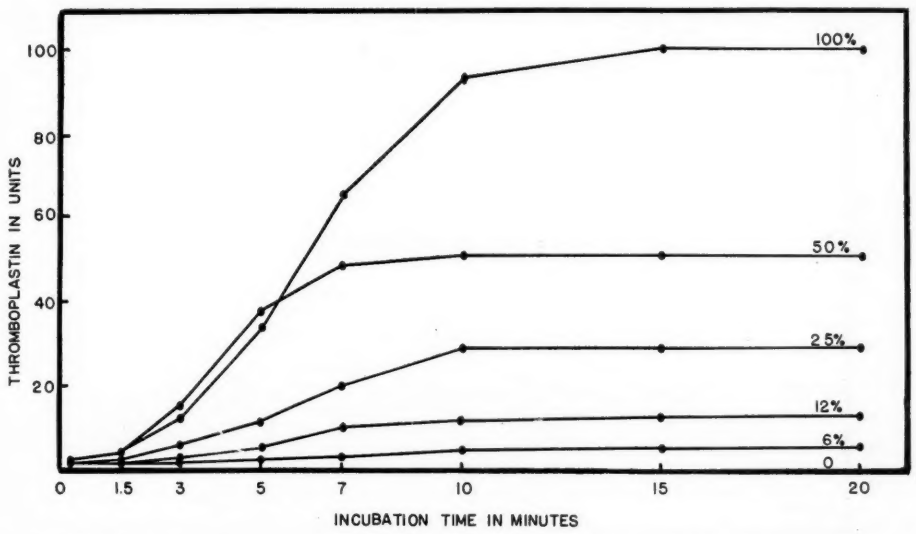

Fig. 4.—Effect of varying concentrations of triturated platelets in the basic reaction mixture.

suggests that PTC acts as an enzyme. Although, theoretically, one would expect varying concentrations of enzyme to give the same yield eventually, the known lability of the reaction mixture may explain why such a result is not observed.

3. AHG. When the concentration of AHG is varied and that of PTC, platelets, and calcium kept constant (Fig. 6), the amount of thromboplastin formed is proportional to the concentration of AHG when the lowest curve is used as a base line. Again, the appearance of a moderate amount of thromboplastic activity is seen in the absence of AHG.

It is difficult to draw conclusions from this experiment when it is probable that at least two reactions are involved. Several interpretations seem possible.

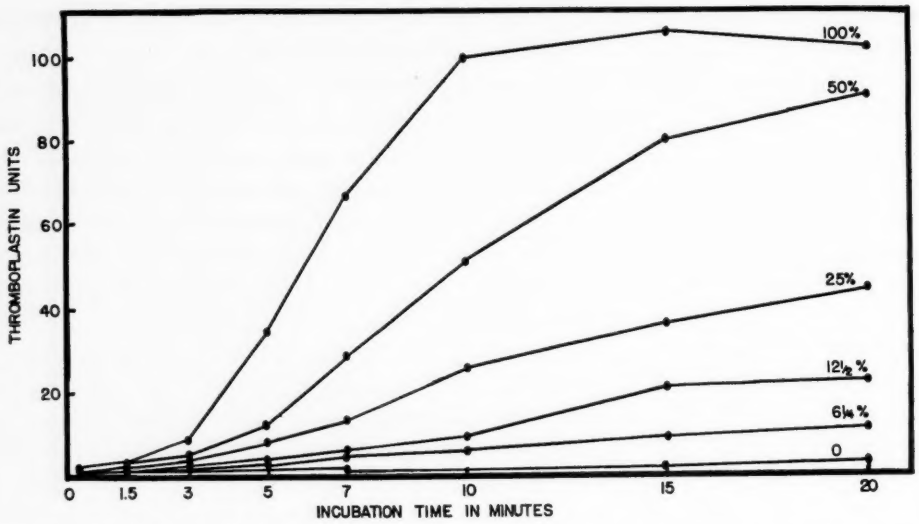

Fig. 5.—Effect of varying concentration of PTC in the basic reaction mixture.

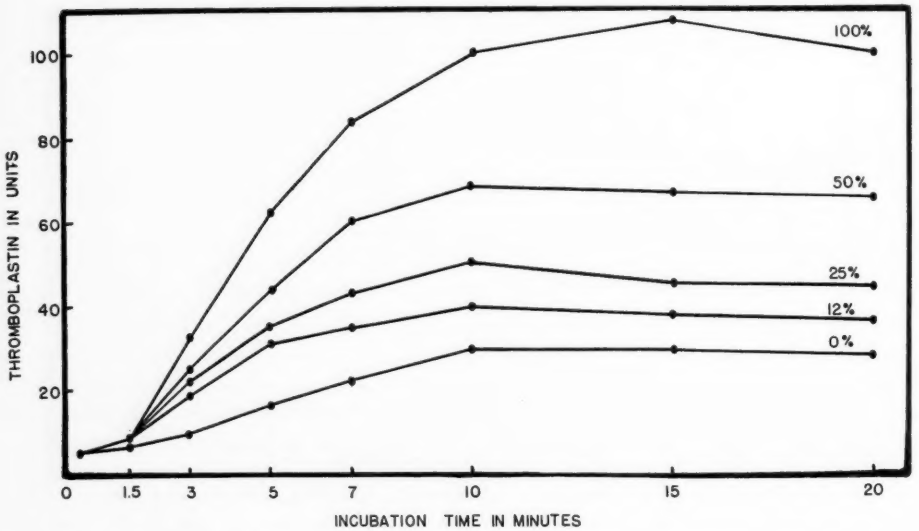

Fig. 6.—Effect of varying concentration of AHG in the basic reaction mixture.

The reaction product of PTC and platelets may combine stoichiometrically with AHG to form thromboplastin or one may serve as a cofactor to the other in prothrombin conversion. The curves are not typical of those which result when the concentration of an enzyme or substrate is varied, though it must be admitted that such curves are conceivable if a very labile enzyme is involved in the reaction.

Effect of Surface.—In the foregoing experiments surface effects have been kept constant. When the surface was varied (Fig. 7), the rate of production and amount of thromboplastin formed were increased by increasing contact

with glass. The only rough surface in the lower curve experiment was that of the glass pipette used to sample the mixture. Comparison of the two upper curves with whole and triturated platelets suggests that this effect is mainly on platelets. In addition, Quick and Epstein[20] and Conley and associates[6] have presented evidence that AHG (thromboplastinogen) is "activated" by rough surfaces.

Effect of Thrombin.—Biggs, Douglas, and Macfarlane[17] have demonstrated the accelerating effect of thrombin on thromboplastin generation. When a small amount of thrombin* is added to the basic reaction mixture, thrombo-

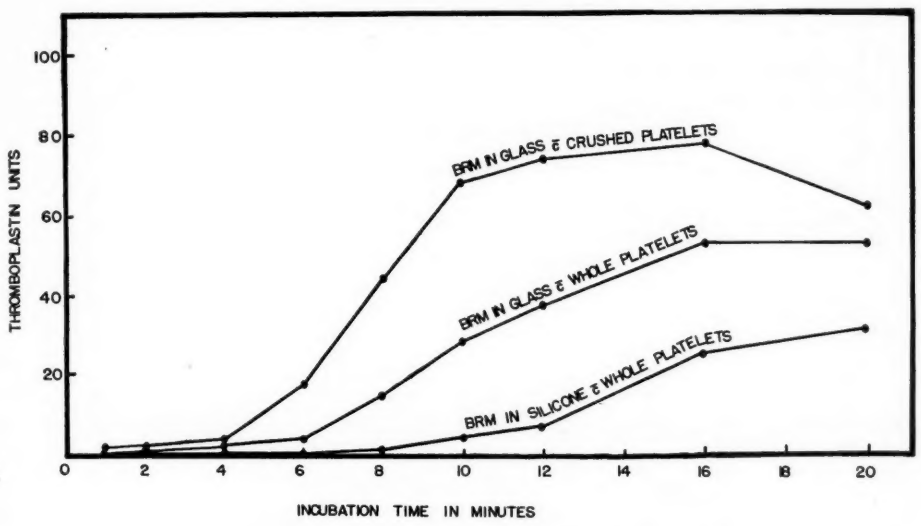

Fig. 7.—Effect of surface on thromboplastin generation.

plastin production is enhanced and the lag phase is eliminated (Fig. 8). This result is obtained with either whole or triturated platelets, and the enhancement appears to be of the same magnitude. The amount of thrombin added in this experiment was not enough in itself to cause a detectable decrease in the clotting time of recalcified plasma.

This experiment sheds no light on the site of action of thrombin in thromboplastin formation. Quick and associates[21] and Stefanini[22] have reported that thrombin has a labilizing effect on platelets. In a later communication, Quick and colleagues[23] suggested that thrombin activates thromboplastinogen (AHG), though the interpretation is not convincing. The results of Desforges and Bigelow[24] seem to indicate that thromboplastic activity develops from thrombin and platelets, either whole or disintegrated. It is not inconceivable that thrombin, like PTC, can react enzymatically with platelets to produce thromboplastic activity.

Antithromboplastin.—When serum was used as a source of PTC in the basic reaction mixture, powerful thromboplastin evolved quickly, either with

*Topical Thrombin (Bovine), Parke, Davis & Co.

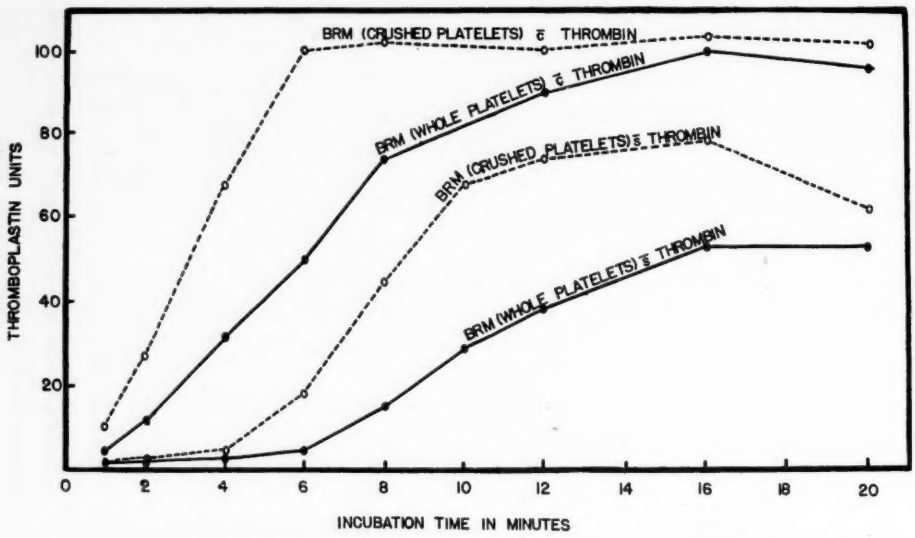
BRM (CRUSHED PLATELETS) ē THROMBIN
BRM (WHOLE PLATELETS) ē THROMBIN
BRM (CRUSHED PLATELETS) ē THROMBIN
BRM (WHOLE PLATELETS) ē THROMBIN
THROMBOPLASTIN UNITS
INCUBATION TIME IN MINUTES

Fig. 8.—Effect of added thrombin on thromboplastin generation.

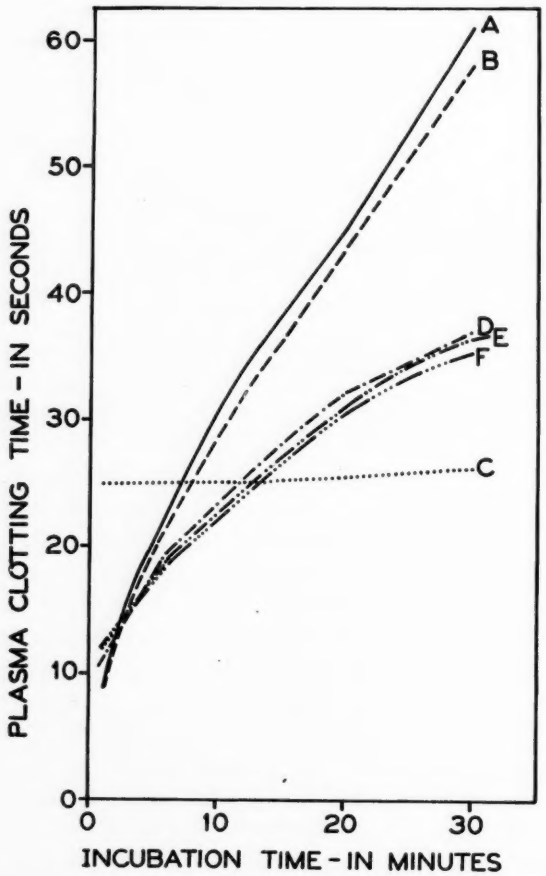

Fig. 9.—Antithromboplastic activity of serum; *A*, serum and saline added to commercial thromboplastin; *B*, serum and AHG incubated together and added to commercial thromboplastin; *C*, saline added to commercial thromboplastin (control); *D*, serum and saline added to spontaneous thromboplastin (made in absence of AHG); *E*, serum and AHG incubated together and added to spontaneous thromboplastin (made in absence of AHG); *F*, serum and saline added to spontaneous thromboplastin (made in presence of AHG).

or without AHG, and then disappeared steadily (Fig. 1, *D* and *E*). Since thromboplastin generated with PTC in the absence of serum is relatively stable, we were led to believe that the effect was due to an antithromboplastin in the serum. At least two substances with this property have been studied by Thomas,[25] Lanchantin and Field,[26] and by Tocantins.[8] In order to investigate this possibility further, serum was incubated with spontaneous and with commercial tissue thromboplastin (Simplastin). Antithromboplastic activity was again demonstrated as shown in Fig. 9, *A* and *D*. In the absence of calcium there was no such activity. The incubation of serum with AHG did not decrease its antithromboplastic activity (Fig. 9, *B* and *E*), nor was there any difference in the effect on thromboplastin generated with and without AHG (Fig. 9, *B* and *E*).

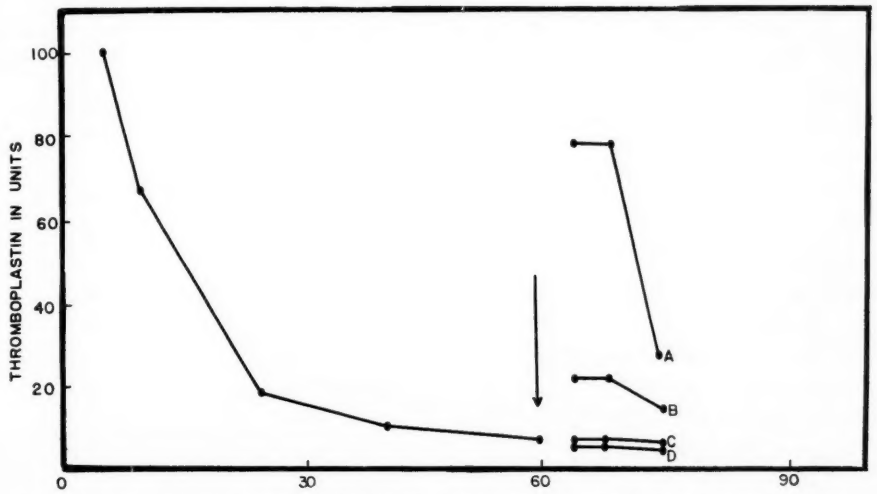

Fig. 10.—Regeneration of thromboplastin. Initial curve obtained by following degeneration of basic reaction mixture in which serum replaced PTC. After one hour mixture was separated into four parts and to each were added: *A*, platelets and AHG; *B*, platelets; *C*, AHG; *D*, PTC.

In the experiment shown in Fig. 10, a reaction mixture was prepared with platelets, AHG, calcium, and serum (as a source of PTC and antithromboplastin). Thromboplastic activity developed rapidly and then steadily diminished until there was very little activity at one hour. The reaction mixture was then divided into four parts and to each was added either PTC, AHG, platelets, or platelets suspended in AHG. These second mixtures were again tested with platelet-poor plasma for reappearance of thromboplastic activity. With PTC or AHG alone (C and D) there was none, but with platelets about 20 thromboplastin units were formed (B), and with platelets suspended in AHG there were almost 80 (*A*). It is of interest to note that in earlier experiments (Figs. 3 and 6) from 20 to 30 thromboplastin units were formed in the absence of AHG in the basic reaction mixture while 90 to 100 units were formed in its presence.

The present experiment affords good evidence that a large amount of PTC remained in the reaction mixture after thromboplastin had been formed

(and destroyed) and, furthermore, that AHG and platelets were consumed in the reaction. These observations lend support to the concept that PTC acts enzymatically on a platelet substrate and that the reaction product combines with AHG to produce thromboplastin.

DISCUSSION

Based on the data presented here, as well as on certain observations by others already cited, the following concept of thromboplastin formation seems worthy of further consideration. As platelets release their factor(s), PTC acts enzymatically on the liberated factor in the presence of calcium to form a weak thromboplastin. AHG then reacts either stoichiometrically with the weak thromboplastin to produce a "complete" thromboplastin or one serves as a cofactor to the other. Thrombin catalyzes the reaction, perhaps by its action on platelet factor. Tissue thromboplastin likewise acts as a catalyzer by causing thrombin formation. Accumulation of thromboplastin is limited by normally occurring antithromboplastin(s).

Surface effects other than the probable one on platelets are likely. Several investigators[6, 20, 27] have presented data suggesting activation of thromboplastin precursors by rough surfaces. Epstein and Quick[28] have shown that the injection of a potent platelet extract into rabbits does not produce a demonstrable effect on the coagulation system. If the afore-mentioned concept of thromboplastin formation is correct, it may be necessary to postulate an inactive precursor of PTC (which would not react with the platelet extract) to explain their results. Whether such a precursor exists remains to be determined. Also of interest are the animal experiments of Wessler,[29] in which it was shown that intravenous injection of a serum fraction prepared by barium sulfate adsorption and citrate elution was capable of producing thrombosis in vein segments isolated after injection, but not in the open circulation. Although the results were attributed to SPCA in the preparation, active PTC was in all probability present in large amounts and could have been the responsible agent.

Whether the activities of thromboplastin generated with and without AHG differ qualitatively as well as quantitatively remains to be determined. Two previous observations arouse speculation. In the thromboplastinogen activity test of Quick and associates,[30] a labile component is destroyed when tissue thromboplastin is heated and the resulting preparation will correct the diminished prothrombin consumption of thrombocytopenic, but not of hemophilic, blood. Langdell, Wagner, and Brinkhous[31] have developed the concept of "partial" and "complete" thromboplastins and have shown that the "prothrombin time" with hemophilic plasma is prolonged using partial thromboplastins. Further studies are in progress in an attempt to clarify these relationships.

The identity of the platelet cofactor of Johnson[32, 33] requires elucidation. This substance, which could be extracted from either normal or hemophilic plasma or serum, seemed to serve as a cofactor with platelet particles

to develop thromboplastic activity. Biggs and Douglas[16] concluded that Christmas factor (PTC), AHG, factor V,[34] factor VII,[35] calcium, and platelets are all required for the generation of a "complete" thromboplastin. Brain thromboplastin was considered incomplete in that it requires factors V and VII for adequate prothrombin conversion. That they did not find PTC, AHG, platelets, and calcium sufficient appears to have been due to differences in terminology and concept rather than to actual difference in results.

SUMMARY

Plasma thromboplastin component, platelets, antihemophilic globulin, rough surfaces, and calcium are required for maximum thromboplastin production in vitro. The yield is dependent quantitatively upon these factors and is increased by at least one additional factor, thrombin. An antithromboplastic factor present in normal serum rapidly inactivates thromboplastin.

A concept of thromboplastin formation is described in which plasma thromboplastin component acts enzymatically on platelet factor(s) to release a substance which then combines with antihemophilic globulin to form thromboplastin.

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ELECTROPHORETIC STUDIES OF THE PLASMA PROTEINS IN VIRUS HEPATITIS

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INTRODUCTION

ELECTROPHORETIC studies on serum proteins in virus hepatitis have been reported previously by several workers.[1-3] Since some cases of virus hepatitis eventually result in chronic liver disease,[3] it would be of interest to determine the severity of the infection through a study of the changes that occur in the plasma protein pattern.

Recently, there was an outbreak of virus hepatitis in one of the textile mills in Bombay, and most of the afflicted patients were admitted to the Infectious Disease Hospital. The present investigation was undertaken to determine the plasma protein pattern during the early phase of the disease and, if possible, to correlate the protein pattern with the severity of the disease.

MATERIALS AND METHODS

Thity-five male patients out of more than one hundred admitted to the hospital were available for our studies. Five of the patients had no jaundice while the rest showed icterus indices varying from 20 to 400. All patients were discharged after a few weeks of observation and were not available for follow-up study. The plasma protein patterns of ten workers from other mills were studied as controls.

Blood was collected in the morning by venepuncture and put into a vial containing the oxalate mixture recommended by Wintrobe.[4] Patients did not fast before collection of blood. Plasma was separated by centrifugation. The total protein content was estimated by the specific gravity method using copper sulfate solutions.[5]

Fractionation was carried out by paper electrophoresis as described by Gronwall.[6] Two drops of plasma, each equivalent to 0.01 ml., were placed 2 cm. from the cathode end. Six to eight strips were run simultaneously for eight to twelve hours at 100 to 150 mv. Strips were dried and stained subsequently in 1 per cent bromphenol blue. Five definite fractions could be delineated visually. Each fraction was cut out and the dye eluted in 5 per cent sodium carbonate in 50 per cent methanol. The intensity of the dye was considered to be proportional to the amount of protein and the quantity of each fraction was obtained by computing the product of the per cent of absorbed dye and the total protein content.[7]

Standard deviation and standard error were calculated according to conventional methods. When the difference of two means was more than twice the standard error of the difference, the results were deemed statistically significant.

RESULTS

Table I shows the plasma protein pattern of ten normal volunteers and of thirty-five patients with virus hepatitis grouped according to the severity of

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Received for publication, April 19, 1954.

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the disease estimated on the basis of the icterus index. Figures obtained from twenty-seven cases of portal cirrhosis with ascites have been included for comparison.

TABLE I. PLASMA PROTEIN PATTERN IN VIRUS HEPATITIS AND PORTAL CIRRHOSIS WITH ASCITES

	NO.	ICTERUS INDEX UNITS	LEVEL IN GRAMS PER 100 ML. STANDARD DEVIATION AND STANDARD ERROR					
					GLOBULINS			
			TOTAL PROTEINS	ALBUMIN	ALPHA 1	ALPHA 2	BETA	GAMMA WITH FIBRINOGEN
Normal mill workers	10	–	7.70	4.50	0.33	0.50	0.80	1.70
			.47	.50	.14	.14	.15	.40
			.14	.16	.04	.04	.05	.13
Virus hepatitis	10	0-70	7.40	3.38	0.38	0.49	0.80	2.30
			.60	.30	.22	.18	.40	.18
			.17	.10	.08	.06	.12	.02
Virus hepatitis	13	71-200	7.50	3.50	0.32	0.44	0.83	2.44
			.50	.50	.45	.20	.14	.35
			.14	.14	.04	.05	.04	.10
Virus hepatitis	12	201-400	7.40	3.70	0.17	0.24	0.97	2.40
			.50	.17	.08	.16	.45	.17
			.15	.05	0.2	.04	.14	.05
Portal cirrhosis with ascites	27	–	6.40	2.10	0.20	0.38	0.74	3.00
			.90	.50	.16	.20	.25	.74
			.18	.10	.03	.04	.05	.14

Comparing the plasma protein pattern of the ten normal mill workers with the pattern obtained from 100 medical students,[7] we found the same values for total proteins, albumin, and beta and gamma globulins. The figures for alpha 1 and alpha 2 globulins are slightly higher in the mill workers than in the medical students.

There was a drop in the total protein in cases of virus hepatitis which was not correlated with the severity of the liver damage, and which was less marked than the drop in total protein in cases of portal cirrhosis. The drop in the albumin fraction, however, was much greater than the fall in the total protein. Again, the albumin level was not correlated with the degree of icterus and was not so great as the reduction in albumin level found in portal cirrhosis.

Changes in alpha 1 and alpha 2 globulins are interesting, since a significant drop occurred only in the most severe cases of infective hepatitis and in portal cirrhosis.

There was not much variation in the beta globulin fraction. The gamma fraction, including fibrinogen, showed a definite rise above normal, although there is little difference between the levels in the three groups of infectious hepatitis. In portal cirrhosis, however, there is a more marked rise in the gamma fraction, and in some instances a definite demarcation between gamma 1 and gamma 2 was observed.

The five cases without elevation of icterus index form too small a group for statistical comparison and these cases are included in the table among the cases with an icterus index of 0 to 70. It was noted, however, that these individuals showed no drop in any of the globulin levels, the only abnormality being a fall in the albumin and total protein level.

DISCUSSION

Electrophoretic analysis of the serum proteins in virus hepatitis was first described in 1943 by Gray and Barron,[1] who reported five cases with diminished albumin in all, elevated gamma globulin in four, and elevated beta globulin in two. Franklin,[2] in his report of six cases of virus hepatitis, and Ricketts,[3] in ten cases of virus hepatitis, have confirmed these findings.

In this series of thirty-five cases of virus hepatitis, the drop in total proteins is not so marked as the fall in the albumin fraction, and even this is less marked in hepatitis than in cirrhosis. The drop of the albumin level is a direct result of impaired hepatic function. A similar drop occurs in eclampsia.[8]

It is interesting to note that the drop in albumin was seen even in cases without clinical or laboratory evidence of icterus, and this may be a significant diagnostic criteria of infectious hepatitis.

The alpha 1 and alpha 2 fractions did not show any variation from normal in the mild cases of virus hepatitis. A significant fall in these two fractions occurred, however, in the most severe cases of infectious hepatitis and in cases of portal cirrhosis.

The decrease in these two fractions of globulin is probably indicative of marked derangement of hepatic function, in both acute and chronic conditions. This drop in alpha 1 and alpha 2 fractions has also been noted in cases of eclampsia,[8] where liver damage is probable. This finding was not stressed by previous workers. From Ricketts'[3] figures, however, it is evident that some of his cases showed a fall in the alpha 1 and alpha 2 fractions, usually those cases with a high serum bilirubin content and strongly positive cephalin flocculation test. It is suggested that cases showing a low alpha 1 and alpha 2 globulin should be observed to ascertain whether these changes have prognostic importance.

Previous workers have reported a rise in the beta fraction in some of their cases but not in all. In our series of virus hepatitis and also cirrhosis, there was no rise in the average beta fraction, although we have obtained high figures in some cases of virus hepatitis and some cases of portal cirrhosis.

The well-known rise in the gamma fraction did not occur in the cases without jaundice, but otherwise the extent of the rise in gamma globulin was not correlated with the degree of icterus.

Furthermore, the rise in gamma globulins was not so extensive as in portal cirrhosis, and never resulted in a clear separation of gamma 1 and gamma 2 fractions. Our figures for gamma fraction are higher than those given by workers from other countries, but this is consistent with higher figures we have observed in normal mill workers and previously reported in 100 medical students.[7]

SUMMARY

The plasma protein pattern in thirty-five cases of virus hepatitis has been analyzed and compared with that of normal mill workers and cases of portal cirrhosis.

There was a drop in the albumin fraction which can be detected even in cases without laboratory or clinical evidence of jaundice.

There was a significant drop in alpha 1 and alpha 2 fractions in those cases with severe icterus.

There was no significant change in the average beta fraction although some cases showed a rise.

There was a marked rise in the gamma fraction; less marked than in the portal cirrhosis. This elevation of gamma globulin did not occur in the cases without jaundice.

We wish to express our thanks to the Medical Superintendent, Infectious Diseases Hospital, Bombay, for his cooperation in carrying out this investigation.

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STUDIES OF THE THYMOL TURBIDITY, CEPHALIN FLOCCULATION, AND THYMOL FLOCCULATION TESTS

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THE clinician concerned with the care of workers in scientific laboratories or industry using many new solvents or chemicals of poorly defined toxic potentialities depends on information derived from clinical and laboratory observation of these workers. A proper approach to the assessment of toxic properties of scientifically or industrially useful substances would take into account the probabilities that: (1) the latent period before the appearance of gross and disabling disease might be long; (2) the percentage of individuals afflicted, because of hitherto poorly understood variables, might be small; and (3) that various tests of liver function might be a sensitive indicator of general somatic disease processes even though the organ system primarily affected (as in beryllium poisoning) might be other than the liver, which, however, may also be affected to a lesser degree.

Two approaches may be taken for this evaluation. One concerns the study of the individual by means of regularly recurrent tests of liver function for the detection of definitive deviations from the norms chosen. The other is concerned with statistical analyses of trends of deviations from normality as measured by liver studies in large numbers of workers exposed to hitherto unproved but potentially toxic substances. Both in the evaluation of potentially hepatotoxic substances and in the care of the patient in the hospital it is important that a test of liver funcion yield a minimum of "false positive" results while maintaining sensitivity for the detection of early and minimal deviations from normal. To accomplish this the use of well-established normal standards is imperative. Furthermore, in evaluating the usefulness of a liver function test it must be understood that a very sensitive test will give positive results in a very high percentage of cases with even mild hepatic involvement. It will, however, also give positive results in a fairly large number of clinically normal persons. On the other hand, a less sensitive method will exclude most of the normals and a fair number of patients with liver disease. The most useful test is the one which screens reasonably well in both directions.

The thymol turbidity test in conjunction with the cephalin cholesterol test has given much useful information in the diagnosis and differential diagnosis of various types of liver disorders. Preparatory to the study of screening tests of liver function in groups of people at this laboratory working with new elements and compounds of undetermined hepatotoxic properties, work was done on "normal" individuals for the establishment of a base line. Thymol turbidity values were found to be higher than the upper limit of normal usually accepted for the Maclagen method.[1-3] Because of these and similar reported findings,[4-7] and because of variations in sensitivity of three reagents commonly used for this test,[3, 8, 9] we undertook to compare thymol values obtained with each of these reagents in normal individuals in order to predicate a normal range for this screening test. In addition, similar studies were done on patients of the University of Chicago Clinics, both hospitalized and ambulatory, the majority of whom were suffering from liver disorders,

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Received for publication, Feb. 15, 1954.
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and on a small group of laboratory workers with diagnosed or suspected liver disease. Results were analyzed considering both 3.0 and 5.0 thymol units[7] as the upper limit of normal.

The thymol flocculation and the cephalin cholesterol flocculation tests were performed routinely with the thymol turbidity studies. In the course of this investigation additional data have been accumulated concerning the thymol flocculation test. Because of observed differences in the results of the cephalin cholesterol test with use of two commercial antigens, both within this laboratory and in another laboratory,[10] a comparison was made of cephalin results using both antigens in order to evaluate the sensitivity of each for both normal individuals and patients.

METHODS

1. Three commonly used and one modified thymol reagent were employed. They are: (a) Maclagen thymol reagent as described, at pH 7.72*[8]; (b) thymol reagent as modified by Huerga and Popper, at pH 7.72[9]; (c) Maclagen thymol reagent as modified by Mateer and associates,[3] at pH 7.55; and (d) thymol reagent as modified by Huerga and Popper, at pH 7.55.

All chemicals used were Merck, A. R. The pH of reagents (a) and (b) was not adjusted, but was always pH 7.72, in both freshly made and refrigerated solutions. The pH of solutions (c) and (d), measured with a Beckman pH meter, was adjusted by using necessary amounts of either diethyl-barbituric acid or sodium barbital. Reagents were refrigerated and brought to room temperature before use.

2. Measurements were made with the Evelyn photoelectric colorimeter using the 660 mμ filter. The calculation of thymol turbidity measurements was based on the precalibrated colloidal glass standard prepared and standardized by the Commission on Liver Disease of the Armed Forces Epidemiological Board.[7] All studies were carried out with this standard and previous results were recalculated on the basis of the new standard curve. Intercomparison of method and results on identical samples was done with the Biochemical Laboratory of Michael Reese Hospital (Chicago). All tests were run in duplicate.

3. Six milliliters of the thymol buffer was added to 0.1 ml. of serum in the colorimeter tube, mixed, stoppered, and allowed to stand for thirty minutes at room temperature. The sample was mixed again before reading. Both a serum blank and a buffer blank were used. Final results were determined on readings made with the serum blank (0.1 ml. serum diluted with 6.0 ml. of 0.85 per cent saline solution).

4. The thymol samples stood overnight at room temperature in the dark (eighteen hours) for the thymol flocculation reading.[2]

5. Cephalin cholesterol flocculation tests:

a. The cephalin cholesterol flocculation test was carried out as usual,[11] using 4.0 ml. of 0.85 per cent saline with Merthiolate (sodium ethylmercurithiosalicylate) 1:10,000, 0.2 ml. serum, and 1.0 ml. of antigen. Antigen was prepared daily from the refrigerated stock solution. Both twenty-four- and forty-eight-hour readings were made. Unless otherwise indicated, only the forty-eight-hour reading is reported. Bacto-cephalin Cholesterol Antigen (Difco Laboratories) was used routinely.

b. A comparison was made between results using Bacto-cephalin Cholesterol Antigen (Difco Laboratories) and Cephalin Cholesterol Mixture (Wilson Laboratories). Both stock antigens were prepared according to the manufacturer's specific directions, and the test was carried out as described previously. Different lots of antigen were selected each week in order to obtain proper sampling.

6. Groups studied:

Group I. Argonne National Laboratory workers randomly selected and found to be normal; blood samples taken at time of physical examination.

Group II. Argonne National Laboratory workers and University of Chicago Clinic patients with known liver disease, liver involvement during the course of

*Using several varieties of A. R. chemicals, and following the original method, this laboratory has for three years consistently obtained a pH of 7.72 and not 7.80 for the Maclagen reagent.

a systemic disease, or with a history of liver disease. The diseases and number of patients in each category were as follows: cirrhosis, 24; infectious hepatitis, 19; toxic hepatitis, 5; homologous serum jaundice, 1; hepatitis with cirrhosis, 1; granulomatous disease of the liver, etiology undetermined, 2; Banti's syndrome, 2; carcinomatosis with metastases to the liver, 2; and one patient each with the following: primary carcinoma of the liver, fatty infiltration of the liver, beryllium disease, Von Gierke's glycogen storage disease, obstructive biliary cirrhosis, postoperative stricture of the bile duct, and carcinoma of the pancreas with bile duct obstruction.

Group III. Argonne National Laboratory workers and University of Chicago Clinic patients ill with diseases other than liver disease.

7. Control studies were made during the course of the routine yearly physical examination on randomly selected normal individuals. Postprandial morning serum samples were used. The clot was allowed to form under refrigeration and the thymol and cephalin tests were done immediately after clot retraction.

A complete thymol study included the thymol turbidity test and the thymol flocculation reading, both performed using all four thymol reagents, and the cephalin cholesterol determination.

When higher than usually accepted values were obtained, repeat tests were done on samples collected from the fasting individual. Serum bilirubin determinations were made on all repeat samples and, depending upon the judgment of the examining physician, a bromsulphalein retention test was also performed. At three-, six-, and nine-month intervals, repeat fasting samples were obtained from individuals showing unexplained persistently high values.

Each worker had a complete physical examination, chest roentgenogram, urinalysis, and hematologic and serologic studies consisting of the Kahn test, sedimentation rate, hemoglobin and hematocrit, red cell, white cell, and differential counts.

Hospital patients were from the Gastrointestinal Service, University of Chicago Clinics. Sera from these patients were refrigerated and tests for this study were run twenty-four hours after the samples were drawn. All samples from both Clinic patients and Argonne National Laboratory patients were collected from the fasting individual. Thymol and cephalin tests on the latter patients were performed immediately after clot retraction.

The comparison of the two cephalin cholesterol antigens was a separate study which included both control individuals and patients not necessarily included in the thymol study. A complete cephalin test included determinations with both antigens and, in addition, thymol turbidity and thymol flocculation tests using at least one reagent. Control individuals showing abnormal results were also studied as described previously, including repeat determinations at trimonthly intervals.

8. Number of individuals and number of tests:

	No. of Individuals	No. of Tests
a. Thymol turbidity study		
Group I	130	198
Group II	41	58
Group III	28	35
Total	199	291
b. Thymol flocculation study:		
Group I	130	198
Group II	63	80
Group III	51	57
Total	244	335
c. Cephalin cholesterol study		
Group I	96	118
Group II	31	31
Group III	27	27
Total	154	176

In the following data, the methods will be designated as follows:

Method I, Maclagen thymol reagent at pH 7.72
Method II, Huerga and Popper thymol reagent at pH 7.72
Method III, Maclagen thymol reagent at pH 7.55
Method IV, Huerga and Popper thymol reagent at pH 7.55

Individuals studied will be indicated by group number, as described previously.

RESULTS

I. Comparison of Thymol Test Methods and Comparison of Two Limits of Normal.—

A. Tables I and II: In an analysis of the results using 3.0 thymol units as the upper limit of normal, Table I shows the percentage of normal individuals falling within this range and the percentage of patients giving results above this range. It can be seen that Method II demonstrates the greatest percentage of normal persons while Method III screens the highest percentage of patients with liver disease or involvement. In a statistical comparison of Methods II and IV, application of the t test for determining the significance of the percentage differences shows that while Method II is superior in detecting normal individuals, there is no significant difference between the two methods in detecting patients with liver disease. Using 3.0 thymol units as the upper limit of normal, Method II would seem to be the method of choice as a general method to screen out normal individuals and to detect patients with liver disease. Method II is also somewhat superior in eliminating positive results from patients with diseases other than liver disease. Method I, the Maclagen method at pH 7.72, is in general the least adequate of the methods as a screening procedure for discriminating normal persons from patients with liver disease.

TABLE I. INDIVIDUALS GIVING CLINICALLY CONSISTENT* RESULTS WITH TEST METHOD: PERCENTAGE OF CORRECT CLASSIFICATION

TEST METHOD	I	II	III	IV
Group I	72.9	90.6	47.3	76.5
Group II	75.6	80.0	92.6	87.5
Group III	53.8	40.0	65.4	62.5

3.0 thymol units upper limit of normal.

*The phrase "clinically consistent results" refers to results below 3.0 thymol units for normal individuals and to results above 3.0 thymol units for patients.

TABLE II. INDIVIDUALS GIVING CLINICALLY CONSISTENT* RESULTS WITH TEST METHOD: PERCENTAGE OF CORRECT CLASSIFICATION

TEST METHOD	I	II	III	IV
Group I	93.0	96.6	88.4	92.4
Group II	53.7	45.0	70.7	62.5
Group III	19.2	40.0	38.4	25.0

5.0 thymol units upper limit of normal.

*The phrase "clinically consistent results" refers to results below 5.0 thymol units for normal individuals and to results above 5.0 thymol units for patients.

Comparably, Table II shows the same data using 5.0 thymol units as the upper limit of normal. With this limit all four test methods exhibit a greater sensitivity in screening out normal individuals, but less sensitivity in detecting patients with liver impairment. Again, Method II is superior to the other tests in demonstrating normal persons.

Application of the t test to a statistical comparison of Methods III and IV shows that Method IV is superior in detecting normal persons, while there is no significant difference between the two methods in determining patients with liver disease. Also, in this situation, Method I is as adequate as Method IV. Thus, considering 5.0 thymol units as the upper limit of normal, Method IV would be the method of choice.

A study of the percentage distribution of thymol values in all four tests, regardless of the test method used, combined with results obtained from Tables I and II, presents the following picture:

Group I

1. 90.6 per cent of normal individuals give results less than 3.0 thymol units by one method.
2. An additional 6.0 per cent of individuals give results by this same method falling between 3.1 and 5.0 thymol units. Of this group 5.1 per cent give results above 5.0 thymol units by three of the four methods; only 0.9 per cent of individuals give results between 3.1 and 5.0 thymol units by all four test methods.
3. 3.4 per cent of normal individuals show values above the 5.0 thymol limit of normal, regardless of test method used.

Group II

1. 70.7 per cent of patients with liver disease give thymol values above 5.1 units by one method; 45 per cent give values above this level regardless of test method used.
2. 21.9 per cent of patients with liver disease give results in the range of 3.1 to 5.0 thymol units by at least one test method.
3. 7.4 per cent of patients with liver disease will give values below 3.0 thymol units, regardless of method used.

B. Table III: The percentage distribution of thymol values has been included in order to demonstrate the range of these values. Considering both normal individuals and patients, the range of values is most compact with Method II and most extended with Method III.

TABLE III. DISTRIBUTION OF THYMOL VALUES: PERCENTAGE OF TESTS

	TEST METHOD	0-3.0	3.1-5.0	5.1-5.5	5.6-6.0	6.1-7.0	7.1-8.0	8.1-9.0	9.1-10.0	10.1-12.0	12.1-14.0	OVER 14.1 UNITS
Group I	I	66	21	02	025	025	03	02	01	—	—	—
	II	79	11	02	03	—	03	01	01	—	—	—
	III	36	41	03	02	04	02	05	02	03	01	01
	IV	68	17	02	02	05	02	—	—	04	—	—
Group II	I	15	30	—	—	11	15	11	04	07	—	07
	II	22	30	15	08	07	07	—	07	—	—	04
	III	04	19	08	07	08	07	11	07	15	07	07
	IV	11	18	05	13	16	13	05	11	05	—	03
Group III	I	40	30	10	—	—	10	—	—	—	—	10
	II	80	—	—	—	10	—	—	—	—	10	—
	III	30	40	—	—	10	—	—	10	—	—	10
	IV	40	40	—	—	—	10	—	—	—	10	—

This table has been constructed on the basis of values for total number of thymol tests, with no correction made for repeated tests on certain individuals. It therefore does not numerically correspond with results expressed in other tables in which correction has been made for repeated tests.

II. Thymol Flocculation Studies.—

A. Thymol Flocculation Results in Relation to Method (Table IV): With normal individuals there is a decided increase in thymol flocculation as the pH of the test solution decreases. With both groups of patients there is a significant difference in test results only between Methods I and IV, the other differences being close to the range of the standard error.

TABLE IV. THYMOL FLOCCULATION RESULTS IN RELATION TO METHOD: PERCENTAGE OF TESTS

TEST METHOD	I	II	III	IV
		Group I		
Flocc.				
0 to 1+	89.0	90.9	78.6	81.8
2+ to 4+	11.0	9.1	21.4	18.2
		Group II		
Flocc.				
0 to 1+	65.8	60.0	53.6	48.0
2+ to 4+	34.2	40.0	46.4	52.0
		Group III		
Flocc.				
0 to 1+	63.0	50.0	63.0	41.7
2+ to 4+	37.0	50.0	37.0	58.3

Regardless of test method, statistical analysis of the results shows that the thymol flocculation test discriminates fairly well for normal persons and discriminates poorly for patients, either those with liver impairment or those with other diseases. About one-half of the patients with liver disease give a result of 0 to 1+ flocculation, the usually accepted limits of normal.

B. Correlation of Thymol Flocculation Tests With Thymol Turbidity and Cephalin Cholesterol Flocculation Tests: In an attempt to find some correlation between the thymol flocculation test on the one hand, and the thymol turbidity and cephalin cholesterol tests on the other, a statistical analysis was made, the results of the thymol turbidity test being classified for this purpose on a 0 to 4+ basis. Some individuals were consistently 3 to 4+ in all three tests (Group I, 2 per cent, Group II, 15 per cent, Group III, 7 per cent). Otherwise no correlation was found between the thymol flocculation and thymol turbidity tests. In patients with liver disease, an association existed between 2 to 4+ thymol flocculation results and 4+ cephalin cholesterol results. Seventy per cent of individuals with thymol flocculation results of 2 to 4+ gave cephalin results of 4+. In normal individuals with thymol flocculation results of 2 to 4+, 54 per cent also gave cephalin cholesterol results of 2 to 4+. There was little correlation between the abnormal thymol flocculation results of patients with other diseases and abnormal cephalin cholesterol results.

Thymol flocculation results were also correlated with the clinical diagnosis. Of sixty-three patients with liver disease or involvement, 40 per cent showed 2 to 4+ thymol flocculation. Twelve of these individuals had hepatitis and ten had cirrhosis. Of these same patients with a normal thymol flocculation, fourteen had hepatitis and fourteen had cirrhosis. One patient with carcinoma of

the pancreas with complete bile duct obstruction showed an abnormal thymol flocculation; two others with carcinomatosis with metastases to the liver showed normal flocculation.

Results with patients with other diseases were comparable and equally inconclusive. As with patients with liver disease or involvement, there was no clear correlation between clinical diagnosis, stage of disease, and the thymol flocculation result.

III. Comparison of Cephalin Cholesterol Antigen (Table V).—

It can be seen that Antigen B gives 0 to 1+ results with 90 per cent of normal individuals, in comparison with 77 per cent given with Antigen A. Results with patients with liver disease, however, are approximately the same with both antigens. With patients ill with other diseases, Antigen B again gives a smaller percentage of 2 to 4+ results. In both groups of patients there are fewer individuals in the 4+ class with use of Antigen B, those falling in the 2+ and 3+ groups making up the difference.

TABLE V. COMPARISON OF CEPHALIN CHOLESTEROL ANTIGENS A AND B*; PERCENTAGE OF INDIVIDUALS (48-HOUR READING)

	ANTIGEN A					ANTIGEN B				
	0	1+	2+	3+	4+	0	1+	2+	3+	4+
Group I	66	11	09	09	05	86	04	06	02	02
Group II	20	03	—	15	62	23	06	06	26	39
Group III	52	07	11	11	19	59	15	07	15	04

*Antigen A is Bacto-cephalin Cholesterol Antigen (Difco Laboratories); Antigen B is cephalin cholesterol mixture (Wilson Laboratories).

IV. Use of Serum Blanks.—

For the entire study both serum and buffer blanks were used in order to eliminate, if possible, some of the interferences usually found with lipemic serum.[14, 16] With both normal individuals and both groups of patients it was found, as might be expected, that in general a higher thymol value was obtained with the use of the buffer blank, this difference being from 0.2 to 1.5 thymol units. Differences greater than 0.5 thymol unit were always found with grossly lipemic sera. Slightly lipemic sera acted as nonlipemic sera did, giving no differences greater than 0.5 thymol unit.

Grossly lipemic sera were not associated per se with a high thymol value. With both normal individuals and some patients with either liver disease or other disease who gave normal thymol values, such values were obtained even though the fasting serum was lipemic. Some patients with liver disease who were followed over a period of several months showed a pattern of thymol turbidity values that was indepenedent of the lipemic state of the sera.

Of 200 tests performed on nonfasting normal individuals after breakfast, 63 people (31 per cent) had visual evidence of slight lipemia. The sera from eleven other individuals showed gross lipemia. Forty-seven of these people (63 per cent) gave results below 3.0 thymol units; fifteen people (21 per cent) with values above this level gave the same results with fasting samples drawn

the following week. Twelve individuals (16 per cent) who gave results greater than 5.0 thymol units gave results below 3.0 thymol units when a fasting sample was drawn.

DISCUSSION

In the evaluation of the state of health of persons convalescent from acute liver disease, the history, physical findings, and clinical course are aids which are not present in the screening of a predominantly healthy group of individuals. For the purpose of following the clinical course of such convalescents the absolute level of normalcy of a test is not as important as its sensitivity for demonstrating slight changes in liver function. For this a sensitive test that eliminates few normal individuals would still be adequate. In the general work of either a hospital or industrial laboratory, however, the test that will screen the largest proportion of normal individuals while maintaining sensitivity for slight degrees of liver impairment would be the method of choice.

Differences in the sensitivity of various reagents used for the thymol test[3, 8, 9] as well as differences in the turbidity standards used for calibrating photoelectric photometers have led to some confusion in the interpretation of thymol turbidity values, and in the introduction of several limits of normal to be used in considering thymol results.[1-7] The recent introduction of a precalibrated colloidal Pyrex glass standard by the Commission on Liver Disease of the Armed Forces Epidemiological Board, under the direction of Dr. J. G. Reinhold,[7] should largely eliminate the source of error caused by divergent standards.

Although a combination of factors influences protein flocculation tests, in the thymol test the pH, ionic strength of the solution, and the concentration of thymol in the reagent are the most important.[8] It has further been shown[9] not only that thymol concentration varies in reagents prepared according to the traditional methods, but also that turbidity is proportional to the concentration of thymol in the reagent. Thus a reagent with constant thymol concentration was presented.[9] Decreasing the pH of the solution will cause increased turbidity[8, 12]; on this basis the pH of the original Maclagen reagent was decreased.[3] It might be expected that the combination of a stabilized thymol reagent at a more sensitive pH would give an improved test with enhanced clinical significance. For this reason the Huerga and Popper reagent at a pH of 7.55 was used.

Results shown in Tables I and II indicate that although some greater sensitivity in screening normal individuals is obtained by use of 5.0 thymol units as the upper limit of normal, use of 3.0 thymol units as the normal limit gives better selectivity in detecting disease. Since the difference in sensitivity for determining normal individuals is small (6 per cent) with the method that detects the greatest percentage of these normals (Method II), it would seem that the use of 3.0 thymol units as the limit of normal is indicated. Using this limit of normal, the results also show that Method II, the reagent with the stabilized amount of thymol, screens the greatest number of normal individuals and is only slightly less sensitive than Method III for determining patients

with liver disease. Method II is also somewhat superior in eliminating positive results from patients with diseases other than liver disease.

With the use of the precalibrated Pyrex glass standard, it can be seen that a large percentage of patients with liver disease or involvement fall in the range of 3.1 to 5.0 thymol units even with use of Method III, which is the most sensitive for detecting such patients. With Method III, 21.9 per cent of patients fall in this range. Even though this pertains, when Method III is used, an upper limit of 5.0 thymol units must be assumed in order to obtain the most adequate information. Whether 3.0 or 5.0 thymol units is used as the upper limit of normal will depend to a large extent on the method employed in each laboratory. Laboratories using Method IV would do better to use 3.0 thymol units as the upper limit of normal.

It will be noted in Table III, which shows the distribution of thymol values, that with the use of Method II the distribution is more compact and the range is contracted in comparison with the results given by the other methods. This is especially significant in the results from normal individuals. In the patients with liver disease (Group II), however, a significant percentage still gives results falling in the range of higher values, indicating that the test has adequate sensitivity. It should be remembered that in liver disease there is poor correlation between the degree of turbidity and the severity of the disease.[1] An ideal turbidity test would give values proportionate to the severity of the disease. In lieu of this, a test that affords sufficient sensitivity to follow the course of hepatocellular disease can be used.

With the use of a controlled standard such as the colloidal glass standard and a method such as Method II which gives a contracted range of values, it becomes possible to place increased confidence in an absolute value of normal. An individual result formerly considered in the "borderline" and indefinite category can therefore have enhanced significance in the evaluation of the patient. Using these tightly controlled standards of normal, it also becomes feasible in the study of large numbers of workers in an industrial environment to note trends toward the development of abnormality of hepatic function.

Regardless of test method used, a minimum of 3.4 per cent of normal individuals studied gave results significantly above 5.0 thymol units. Careful clinical study of these individuals over a nine-month period failed to reveal evidence of hepatic disease. With the use of a calibrated standard and a method that discriminates well for normal individuals, a certain percentage still falls in the abnormal range. This corresponds with results consistently found by others working with large numbers of normal individuals.[4, 5] (Some discrepancy in percentage is probably the result of the difference in thymol reagent used.)

As would be expected in a protein precipitation reaction, there is an increase in thymol flocculation as the pH is lowered. With normal individuals, this increase is significant; in order to discriminate best for normal individuals, Method II should be used. Thus in any report of normal (0 to 1+) and abnormal results, the pH of the thymol solution would have to be considered.

Regardless of test method and increase in flocculation with decrease in pH, statistical analysis shows that this test discriminates poorly for both groups of patients. Further analysis of results, as described previously, showed no clear-cut correlation between clinical diagnosis, stage of disease, and the thymol flocculation result.

The thymol flocculation test has been shown to be an indicator of residual hepatitis and to correlate with the cephalin cholesterol flocculation test.[2, 13, 16] Considering the results found in this study, the value of the test as a screening test of liver function is dubious. Without a liver biopsy as evidence of cellular damage, a positive result in an individual who is otherwise normal would not have much significance. It is possible that future work might prove the thymol flocculation test to be of value either in following specific diseases or as an indicator of change in the liver in response to a specific irritant. Except for the study of chronic, nonicteric hepatitis,[2] in the interest of time and economy it might be well to eliminate this test from the daily routine of the laboratory.

The comparison of the two cephalin cholesterol antigens was carried out over a six-month period and included use of several lots of each antigen in order to avoid the possibility of obtaining any one hypersensitive lot. The results are clear, showing that Antigen A (Difco) gives a significant percentage of positive results in otherwise normal individuals. Antigen B (Wilson) gives fewer positive results for both normal people and patients ill with diseases other than liver disease, while retaining sensitivity for detecting patients with liver disease or involvement.

Since the lipoproteins definitely influence the thymol turbidity test,[4, 14, 15, 16] it is apparent that fasting samples should be obtained as a general rule. When large-scale screening studies are done, thus precluding the use of fasting samples, the results show that it is still possible to have confidence in samples obtained from the nonfasting individual. Thus only a small percentage of normal individuals will have to be recalled because of false reactions caused by the transient lipemia resulting from food intake. The use of a serum blank also eliminates some of the higher values obtained from lipemic serum, regardless of the cause of the lipemia, and is to be recommended for general use.

SUMMARY

A study of four thymol buffer reagents using a precalibrated Pyrex glass standard shows that the best two-way sensitivity for screening both normal individuals and patients with liver damage is obtained by using the Huerga and Popper thymol buffer reagent at pH 7.72 with 3.0 thymol units considered as the upper limit of normal.

Using this standard and this method, the range of thymol values is condensed, giving significance to an absolute value of normal.

Statistical analysis of thymol flocculation results shows that regardless of test method used this test distinguishes normal persons well but shows poor selection of patients with liver impairment or with other diseases.

Correlation of thymol flocculation results with clinical diagnosis for both patients with liver disease and patients with other diseases showed no clear correlation between clinical diagnosis, stage of disease, and the thymol flocculation result.

A commonly used cephalin cholesterol antigen (Difco) was found to give a significant percentage of positive results in normal individuals, and was no more sensitive than another antigen as an indicator of damage in patients with diagnosed or suspected liver disease. Another antigen (Wilson) gives fewer positive results for both normal people and patients ill with diseases other than liver disease, while retaining sensitivity for detecting patients with liver disease or involvement.

The use of serum blanks generally compensates for some of the nonspecific turbidity often found with lipemic sera.

We should like to acknowledge the technical assistance of Betty M. VanDolah.

We also wish to thank Dr. Clarence Cohn, Director, Department of Biochemistry, Medical Research Institute, Michael Reese Hospital, for his interest and his assistance in this study and in reading the manuscript, and Drs. Joseph B. Kirsner and Arthur P. Klotz of the University of Chicago Clinics for making clinical material available to us.

Statistical analysis of the data was performed by Mr. Sylvanus A. Tyler and Miss Joan Gurian, Division of Biological and Medical Research, Argonne National Laboratory.

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THE LIPID INHIBITOR OF BRAIN: MECHANISM OF ITS ANTICOAGULANT ACTION AND ITS COMPARISON WITH THE SOYBEAN INOSITOL PHOSPHATIDE INHIBITOR

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THE crude cephalin fraction of brain is an acetone-insoluble, ether-soluble, lipid mixture which has been studied primarily for its thromboplastic properties.[1-4] However, this preparation also contains an anticoagulant which must be diluted out or removed before the thromboplastic effect can be demonstrated. This lipid inhibitor, which is probably the same anticoagulant as in McLean's[2] original cuorin and heparphosphatid fractions, in 1916, has been studied extensively by Tocantins and his co-workers.[5, 6] They have presented methods for its separation and assay from brain and plasma. They have also shown that this inhibitor is definitely not heparin, for it does not appear to contain carbohydrate, it is not inactivated by protamine, it has no antithrombic activity, and it is unable to block the activation of prothrombin by Russell viper venom.

Overman and Wright[7, 8] described an inhibitor present in the inositol phosphatide fraction of brain, lung, plasma, and soybean phosphatides which seemed to be identical to the inhibitor of Tocantins. The preparation from soybean was said to show approximately the same activity as preparations from brain and lung. This soybean inositol phosphatide inhibitor should not be confused with the soybean trypsin inhibitor which is a protein and which also has been shown to inhibit the clotting reaction.[9]

Both Tocantins and Overman believed their inhibitors to act as antithromboplastins, since their addition to plasma greatly prolonged the clotting times obtained on the subsequent addition of tissue thromboplastin and calcium. An inhibition of proconvertin (Factor VII), convertin (the reaction product between tissue thromboplastin and proconvertin), proaccelerin (Factor V), accelerin (the increased accelerator activity produced upon the addition of a small amount of thrombin to proaccelerin), or of prothrobin itself, however, could produce the same lengthening of the clotting time. In fact, the lipid inhibitor has been reported to lower both plasma prothrombin and proaccelerin activity in dogs and rabbits in vitro and in vivo.[10]

The present experiments are a further attempt to define the mechanism of action of the lipid inhibitor in crude human brain cephalin preparations and to compare its activity with that of a preparation of soybean inositol phosphatide inhibitor. The latter was kindly given to one of us (P. A. O.) by the

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Received for publication, April 12, 1954.

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late Dr. R. S. Overman. The experiments demonstrate that these two inhibitors behave differently. The lipid inhibitor of brain is found to block the reaction between tissue thromboplastin and proconvertin to form convertin, but is ineffective in the presence of preformed convertin. The soybean inhibitor, however, is revealed to remain fully active in the presence of preformed convertin.

MATERIALS AND METHODS

The Lipid Inhibitor of Brain.—This inhibitor was investigated as it existed in the crude cephalin fraction of human brain. This was prepared according to the technique described by Milstone,[4] which is essentially the same procedure as the one used earlier by Eagle.[3] Two hundred grams of brain was washed free of membranes and blood and ground to a fine powder by trituration with 6 changes of 150 ml. of acetone. The powder was then extracted overnight at room temperature with 900 ml. of ether. The ether was evaporated to dryness by suction at 35° C. The residue was washed twice for fifteen minutes in 450 ml. of acetone, and then redissolved in 100 ml. of ether. This was evaporated to dryness and suction continued for seventy-five minutes to remove the last traces of ether. About 4.5 grams of waxy, cream-colored to brown material was obtained. This was then suspended in 100 ml. of Veronal buffer, in which it formed a heavy milky-white suspension which contained some dark-brown particles. The suspension was centrifuged for twenty minutes at 1,700 r.p.m., and again for ten minutes at 2,500 r.p.m. The resultant precipitate consisted of a lower layer containing the brown particles and an upper layer of white particles coarser but otherwise similar to those in the supernatant. This precipitate was discarded. The supernatant, which consisted of a 2.2 Gm. per cent fine milky-white lipid suspension in Veronal buffer, was divided into small aliquots and stored frozen at -20° C. until used.

The inhibitor in this crude cephalin fraction differs from the more purified preparations of Tocantins in that we did not attempt to separate the "cephalin" from the inhibitor by their differential solubilities in cold methyl alcohol. Our material, when added undiluted to platelet-poor plasma, however, had no thromboplastic effect, but instead gave longer clotting times than a control with buffer. As with Tocantins' more purified preparations, it greatly prolonged the clotting time of plasma to which saline brain extract had been added. The inhibitory effect was no longer detectable when the suspension was diluted 1 to 30 with Veronal buffer. The diluted material then exhibited an incomplete thromboplastic action comparable to that of platelet extracts and would clot platelet-poor, glass-activated plasma in about fifty seconds.

Soybean Inositol Phosphatide Inhibitor.—A sample of soybean phosphatide inhibitor supplied in 1952 by the late Dr. R. S. Overman was used. It had been stored as a fine white powder at room temperature for approximately two years. It was dissolved in Veronal buffer to make a 120 mg. per cent solution of neutral pH.

Saline Brain Extract Thromboplastin.—A saline-buffer suspension of human brain was prepared according to methods previously described.[11]

Russell Viper Venom.—The preparation Stypven,* was used. A 1/20,000 solution was prepared by dissolving 0.1 mg. of dried venom in 2 ml. of the distilled water diluent supplied with the venom.

Plasmas.—Nine volumes of venous blood were collected in glass or in silicone-coated tubes containing 1 volume of 0.1 M sodium citrate. *Platelet-poor plasma* was obtained by centrifugation at 12,000 r.p.m. for forty minutes. *Glass-activated plasma* was prepared by adding approximately 1 volume of fine quartz glass powder to 4 volumes of platelet-poor plasma. The plasma was shaken frequently over a fifteen-minute period and the glass powder then removed by centrifugation at 2,500 r.p.m. for five minutes. *Proconvertin-deficient plasma* was obtained from a patient with a congenital deficiency of this factor. This plasma has a normal content of prothrombin and proaccelerin but only about 1 per cent proconvertin. Citrated

*Burroughs Wellcome & Co. (U. S. A.), Inc.

plasma was stored in small lots at -20° C. and defrosted as needed. The clotting behavior of this plasma has been described in detail previously.[12] *Proaccelerin-deficient plasma* was prepared from a patient with parahemophilia[13] and was also available as citrated frozen plasma which was defrosted as needed.

Sera.—Prothrombin-free serum was prepared by the addition of 0.3 ml. of saline brain thromboplastin to each 10 ml. of blood immediately after withdrawal, followed by vigorous stirring for about ten minutes. The clotted blood was allowed to stand for one hour at room temperature and the serum was then collected and stored for at least seven days at refrigerator temperature to permit the complete disappearance of accelerin and convertin formed during clotting. The *normal serum* which was used in these experiments was then citrated with 1 volume of 0.1 M sodium citrate per 5 volumes of serum and stored at -20° C. until used. Dicumarol serum was prepared in a like manner from a patient with a prothrombin-proconvertin level of 5 per cent. *Hemophilia B (PTC deficiency, Christmas disease) serum* was obtained from a patient with the classical clinical findings of hemophilia but with a coagulation defect which was corrected by hemophilia A plasma, by old stored serum, and by the citrate eluate prepared from barium sulfate adsorbate from normal oxalated plasma. *Adsorbed hemophilial B serum* was prepared by first adding 1 part of 0.1 M sodium oxalate to 5 parts of hemophilia B serum. The oxalated serum was then shaken with 100 mg. of barium sulfate (Baker) per milliliter of serum for ten minutes, and the barium sulfate then removed by centrifugation.

Proaccelerin Reagent.—Citrated ox plasma was passed through a 50 per cent asbestos filter according to a technique which has been published elsewhere.[14] For these experiments the ox plasma was adsorbed a second time with 100 mg. per milliliter of barium sulfate to remove the last traces of prothrombin. Such plasma contains proaccelerin, antihemophilic A factor, and fibrinogen, but is free of prothrombin, proconvertin, or antihemophilic B factor.

Accelerin reagent was prepared by adding a small amount of thrombin to the ox plasma. This must be repeated about three times to remove traces of profibrin which is formed in the ox plasma. Otherwise, this altered fibrinogen will give shorter coagulation times when the reagent is added to clotting mixtures. The ox plasma was allowed to stand for thirty minutes at 37° C. to inactivate the added thrombin. This accelerin reagent will give a clotting time of about seventeen seconds in the one-stage proaccelerin system,[12] as compared to a time of about twenty-six seconds for the proaccelerin reagent.

Thrombin.—The preparation Hemoclaudan-Adrenon, Lövens Kemiske Fabrik, Copenhagen, was used. The dried material was made up in Veronal buffer.

Heparin.—Heparin 5 per cent "A-L", Oslo, was diluted in distilled water to the desired strength.

Veronal Buffer.—A modified Veronal buffer (pH 7.35, 0.154μ) was made by mixing sodium diethyl barbiturate 11.75 Gm.; sodium chloride 14.67 Gm., 0.1 N HCL, 430 ml., and distilled water to 2,000 ml.

All incubations and tests were performed at 37° C. unless otherwise stated.

RESULTS

The Different Behavior of the Lipid Inhibitor of Brain, Soybean Inositol Phosphatid Inhibitor, and Heparin.—Differences between these three inhibitors are revealed by a comparison of the effect of each upon the Quick method clotting time when first saline brain extract and then Russell viper venom is used as the thromboplastin. In this system, 0.2 ml. of plasma, 0.2 ml. of the particular thromboplastin, and 0.2 ml. of the inhibitor are incubated for three to five minutes, and then 0.2 ml. of 0.025 M calcium chloride is added to initiate clotting. The data from such an experiment are given in seconds and are the averages of at least two determinations.

TABLE I. THE EFFECT OF THE LIPID INHIBITOR, SOYBEAN INOSITOL PHOSPHATIDE INHIBITOR, AND HEPARIN UPON THE BRAIN THROMBOPLASTIN AND VIPER VENOM CLOTTING TIMES

THROMBOPLASTIN	BUFFER	INHIBITOR			
		LIPID INHIBIT. 22 MG. LIPID/ML.	SOYBEAN INHIBIT. 1.2 MG./ML.	HEPARIN 5μ/ML.	HEPARIN 10μ/ML.
Saline brain extract	16.5	191.5	36.5	18.5	38.0
Russell viper venom	16.0	10.5	37.5	51.5	1500.0

These results reveal that the lipid inhibitor acts as a powerful anticoagulant when aqueous brain thromboplastin is used, but, in striking contrast, shortens the clotting time with Russell viper venom. This shortening when compared to buffer is due to the known potentiating effect of lipoid upon the action of the venom. Soybean phosphatide inhibitor produced about the same lengthening of the clotting time with saline brain extract as with viper venom. Heparin proved to be a much more powerful inhibitor of the venom than of the brain extract, perhaps due to a partial neutralization of the heparin by other substances in the brain extract.

The effect of the three inhibitors upon the thrombin-fibrinogen reaction was also examined. To 0.4 ml. adsorbed ox plasma, used as the source of fibrinogen, were added 0.2 ml. of the inhibitor and 0.2 ml. of a thrombin solution. The data given in Table II confirm the lack of inhibition of the thrombin-fibrinogen reaction by the lipid and soybean inhibitors in contrast to the complete inhibition produced by the heparin. These two experiments clearly demonstrate that the lipid inhibitor, the soybean inositol phosphatide inhibitor, and heparin act differently upon the clotting process.

TABLE II. THE EFFECT OF THE LIPID INHIBITOR, SOYBEAN INOSITOL PHOSPHATIDE INHIBITOR, AND HEPARIN UPON THE THROMBIN-FIBRINOGEN REACTION

BUFFER	LIPID INHIBIT. 22 MG. LIPID/ML.	SOYBEAN INHIBIT. 1.2 MG./ML.	HEPARIN 10μ/ML.
32.0	28.0	35.0	no clot

The Effect of Preformation of Convertin Upon the Activity of the Lipid Inhibitor of Brain and the Soybean Inositol Phosphatide Inhibitor.—The reaction between tissue thromboplastin and proconvertin to form convertin, which in the presence of accelerin will result in rapid and complete conversion of prothrombin to thrombin, has been described in detail elsewhere.[12] Convertin may be preformed by the incubation of tissue thromboplastin with serum in the presence of calcium, for the proconvertin activity of serum is high.

There were two clues to suggest that the lipid inhibitor might prove ineffective against convertin. The first was the demonstration by Tocantins and Carroll[6] of the decreased effectiveness of the inhibitor if it was added to plasma after clotting had been permitted to begin. The second was the failure of the lipid inhibitor to block the activity of Russell viper venom. We have demonstrated[15] that the clotting activity of viper venom is independent of the proconvertin but not of the proaccelerin content of plasma. In the presence of an optimum amount of platelet or lipoid material, Russell viper venom acts like convertin.

In experiments 1, 2, and 3 of Table III, convertin was preformed by the incubation for one minute of 0.1 ml. of a ⅕ dilution of serum in buffer, 0.1 ml. of saline brain extract, and 0.1 ml. of 0.025 M calcium chloride. Then, in rapid succession, 0.1 ml. of the test substance and 0.1 ml. of normal plasma were added to the incubation mixture and the time was measured from the addition of the plasma to clotting. One can see from a comparison of experiments 1 and 2 that the addition of the lipid inhibitor of brain was without effect in the presence of preformed convertin, whereas experiment 3 shows that the soybean inositol phosphatide remained a powerful inhibitor. In control experiments 4 and 5, the inhibitors were added before the incubation was begun and, as expected, both prolonged the clotting time. In experiments 6 and 7, adsorbed ox plasma, as a source of fibrinogen, was substituted for the normal plasma to demonstrate that the rapid clotting times of experiments 1 and 2 were not due to thrombin formation in the incubation mixture.

TABLE III. THE EFFECT OF THE LIPID INHIBITOR OF BRAIN AND OF THE SOYBEAN INOSITOL PHOSPHATIDE UPON THE ACTIVITY OF PREFORMED CONVERTIN

CONSTANT IN INCUBATION MIXTURE: SERUM + THROMBOPLASTIN			
EXP. NO.	ADDED TO INCUB. MIXTURE	ADDED AFTER INCUBATION	CLOTTING TIME (SECONDS)
1	Calcium	Buffer + plasma	12.0
2	Calcium	Lipid inh. + plasma	11.5
3	Calcium	Soybean inh. + plasma	89.5
4	Lipid inh. + calcium	Plasma	58.5
5	Soybean inh. + calcium	Plasma	68.5
6	Calcium	Buffer + ads. ox plasma	155.5
7	Calcium	Lipid inh. + ads. ox plasma	176.0

Aged serum contains not only proconvertin but also the antihemophilic B factor (PTC, Christmas factor). Saline brain extract thromboplastin contains the equivalent of the antihemophilic B factor, for its addition to hemophilia B plasma gives a normal clotting time. Therefore, it seemed unlikely that the protection afforded against the lipid inhibitor by preincubation of thromboplastin with serum was due to a reaction between thromboplastin and antihemophilic B factor rather than to the formation of convertin. To examine this possibility, however, the effect of preincubation of saline brain extract with various dilutions of normal serum, hemophilia B serum, and Dicumarol serum was compared. This was carried out in a system identical to that described previously. For the sake of brevity, only the data obtained with a 1:10 dilution of serum have been given in Table IV, which shows the effect on the clotting time of addition of buffer after the incubation, lipid inhibitor after the incubation, and lipid inhibitor before the incubation. These data clearly show that incubation of saline brain extract with hemophilia B serum blocks the action of the inhibitor as effectively as incubation with normal serum. In contrast, the incubation of Dicumarol serum (prothrombin-proconvertin level of 5 per cent) provided very little convertin formation at a 1:10 dilution. The reaction between the remaining thromboplastin and the proconvertin in the normal plasma added to initiate clotting was partially blocked

by the lipid inhibitor. This is shown by the large difference between the times for buffer and lipid inhibitor added after incubation. Furthermore, when oxalated hemophilia B serum was adsorbed with barium sulfate its protective effect was lost. Such adsorption of hemophilia B serum removes only proconvertin to our knowledge. One may therefore conclude that it is the reaction between thromboplastin and proconvertin to form convertin which provided the protection against the lipid inhibitor demonstrated in these experiments.

TABLE IV. A COMPARISON OF THE PROTECTION AGAINST THE LIPID INHIBITOR (L.I.) FURNISHED BY INCUBATION OF THROMBOPLASTIN WITH A 1:10 DILUTION OF NORMAL SERUM, HEMOPHILIA B SERUM, AND DICUMAROL SERUM

NORMAL SERUM			HEMOPHILIA B SERUM			DICUMAROL SERUM		
BUFFER AFTER	L. I. AFTER	L. I. BEFORE	BUFFER AFTER	L. I. AFTER	L. I. BEFORE	BUFFER AFTER	L. I. AFTER	L. I. BEFORE
12.9	11.4	61.5	12.0	13.7	42.0	17.1	40.5	49.0

In all of the previously mentioned determinations clotting was initiated by the addition of normal plasma immediately after the lipid inhibitor was mixed with the preformed convertin. The possibility remained that the lipid inhibitor could inactivate convertin over a period of several minutes. Therefore, in some experiments, the lipid inhibitor was added to the incubation mixture after one minute, but the plasma was not added until the fifth minute. This allowed four minutes for the lipid inhibitor to act upon the preformed convertin. Moreover, congenital hypoproconvertinemia plasma was added in place of normal plasma to ensure that only the activity of convertin made from the incubation mixture was measured.

TABLE V. THE EFFECT ON CONVERTIN OF INCUBATION WITH THE LIPID INHIBITOR OF BRAIN

CONSTANT IN INCUBATION MIXTURE: UNDIL. SERUM + THROMBOPL.		ADDED AFTER INCUBATION		CLOTTING TIME (SECONDS)
EXP. NO.	ADDED TO INCUB. MIXTURE	AFTER 1 MIN.	AFTER 5 MIN.	
1	Calcium	Buffer + Plasma	—	11.7
2	Calcium	Lipid inhibitor + Plasma	—	11.8
3	Calcium	Buffer	Plasma	14.2
4	Calcium	Lipid inhibitor	Plasma	18.8
5	Buffer	Plasma + Calcium	—	29.0
6	Lipid inhibitor Calcium	Plasma	—	99.0

These experiments are summarized in Table V. They show that the lipid inhibitor is not able to inactivate preformed convertin over a period of four minutes. The slight lengthening of the clotting time, seen by comparing experiments 1 and 2 with experiments 3 and 4, is believed to be due to the inactivation of convertin by its natural serum inactivators. The fact that the clotting time is longer in experiment 4 than in experiment 3 may be explained by the inhibition of further convertin formation after the first minute by the lipid inhibitor. Both times, however, are considerably shorter than that obtained in experiment 5, in which no inhibitor is added but clotting occurs

without permitting the preformation of convertin, and very much shorter than in experiment 6, where the lipid inhibitor is added directly to the incubation mixture.

The Failure of Preincubation With Proaccelerin or Accelerin To Modify the Activity of the Inhibitors.—There is recent evidence[16, 17] to suggest that proaccelerin or accelerin and tissue thromboplastin or its equivalent in the blood may react together on preincubation. The effect upon the activity of the lipid inhibitor of such preincubation was studied in experiments identical to experiments 1, 2, and 5 of Table III except for the substitution of first proaccelerin reagent and then accelerin reagent for the normal serum in the incubation mixture. No protection against the lipid inhibitor was found.

Since the inositol phosphatide inhibitor retained its activity in the presence of preformed convertin, the effect upon this inhibitor of preincubation of convertin with accelerin was studied. This was carried out on the chance that the soybean phosphatide inhibitor might act to block a possible reaction between convertin and accelerin. The clotting mixture in these experiments consisted of 0.1 ml. each of proconvertin reagent (aged normal serum), accelerin reagent, saline brain extract, and 0.030 M calcium chloride, which were incubated together for three minutes before the addition of 0.1 ml. of soybean inhibitor (1.2 mg. per milliliter) and 0.1 ml. of normal plasma. The clotting times obtained in this experiment were essentially the same as those obtained when the soybean inhibitor was included in the original incubation mixture, and were much longer than those obtained when buffer was substituted for the inhibitor. Thus, preincubation experiments with accelerin had no effect upon the activity of either inhibitors.

Attempts To Decide Whether the Lipid Inhibitor Acts as an Antithromboplastin or as an Antiproconvertin.—Several approaches were used to the question of how the lipid inhibitor blocks the tissue thromboplastin-proconvertin reaction. Conclusive evidence could not be obtained, but our findings in general supported Tocantins' assumption that it acts as an antithromboplastin.

Thus, when aged normal serum was incubated with the lipid inhibitor both in the presence and in the absence of calcium, no reduction of the proconvertin content of the serum could be detected. For example, the incubation of 0.5 ml. normal serum, 0.5 ml. lipid inhibitor, and 0.25 ml. of 0.025 M calcium chloride for five minutes at room temperature gave the same clotting times when a dilution of the mixture was added to a modified proconvertin system (0.2 ml. congenital hypoproconvertinemia plasma, 0.2 ml. adsorbed ox plasma, 0.2 ml. of a 1:5 dilution of the incubation mixture in citrated diluting fluid,

TABLE VI. A COMPARISON OF THE EFFECT OF DILUTION OF THE LIPID INHIBITOR OF BRAIN UPON NORMAL PLASMA AND UPON HYPOPROCONVERTINEMIA PLASMA

PLASMA	BUFFER	DILUTIONS OF LIPID INHIBITOR (22 MG. LIPID/ML.)					
		1/12	1/8	1/5	1/3	1/2	1/1
Normal	19.5	25.5	33.0	46.0	69.0	100	198
Hypopro-convert.	61.0	74.0	83.5	98.5	155	218	270-400*

*Poor clots with end points difficult to read.

0.2 ml. of brain extract, and 0.2 ml. of calcium chloride solution) as when buffer was substituted for the lipid inhibitor in the incubation mixture.

The effect was then determined of varying the dilution of the lipid inhibitor upon the clotting time of normal plasma (prothrombin-proconvertin level of about 90 per cent) as compared with congenital hypoproconvertinemia plasma (proconvertin content of about 1 per cent). In this experiment, 0.2 ml. of the respective plasma, 0.2 ml. of brain thromboplastin, and 0.2 ml. of different dilutions of the lipid inhibitor were incubated for three to five minutes and then 0.2 ml. of 0.030 M calcium chloride was added to initiate clotting. The results of this experiment are shown in Table VI. They suggest that the lipid inhibitor does not inactivate proconvertin. If the increase in the clotting time of the normal plasma from nineteen seconds with buffer to sixty-nine seconds with a 1:3 dilution of the inhibitor were due to a reduction of the proconvertin activity of the plasma from about 90 per cent to less than 1 per cent, it would seem reasonable to expect that even in 1:12 dilution of the inhibitor should be more than sufficient to completely inactivate the 1 per cent of proconvertin in the congenital hypoproconvertinemia plasma. At least one would have expected that the dilute inhibitor solutions would have been proportionately more powerful against the hypoproconvertinemia plasma than against the normal plasma. Also, if the inhibitor acted by competing with thromboplastin for proconvertin, it would seem that its effect should be more striking when the available proconvertin is already greatly reduced. These results therefore are suggestive of an antithromboplastic effect.

It was not possible to determine whether the soybean phosphatide inhibitor functioned as an anticonvertin or as an antiprothrombin. The following experiments, however, suggested that it did not act as antiproaccelerin. When 0.2 ml. of parahemophilia plasma, 0.2 ml. of saline brain extract, 0.2 ml. of buffer, and 0.2 ml. of calcium chloride were mixed, a prolonged clotting time of 150 seconds was obtained because of the proaccelerin defect of this plasma. When 0.2 ml. of soybean phosphatide inhibitor was substituted for the buffer, no clot was formed during a one-hour observation period. There is evidence that our parahemophilia plasma is completely free of proaccelerin. Thus, addition of enough thrombin to clot this plasma does not produce a measurable increase in its activity such as would occur if a small amount of proaccelerin were converted to accelerin. Furthermore, the Quick clotting time of oxalated parahemophilia plasma is not lengthened by storage at 6° C. for fourteen days. A lengthening would be expected if the plasma contained any proaccelerin, since this would be inactivated by the storage. Therefore, we believe that the failure of the parahemophilia plasma to clot upon the addition of the soybean phosphatide inhibitor should be interpreted as an inhibition of some factor other than proaccelerin. This is probably convertin, but possibly prothrombin itself. In support of this explanation is the failure to find any inhibition of proaccelerin activity when 0.4 ml. of ox plasma was incubated for three minutes with 0.2 ml. of soybean inhibitor and 0.2 ml. of calcium chloride and the proaccelerin content of a 1:20 dilution of this mixture was measured in the one-stage proaccelerin system.

DISCUSSION

The discovery of inhibitors which specifically block different stages of the coagulation process would permit an analysis of the clotting reaction by techniques analogous to those used so fruitfully in the identification of the intermediate products of carbohydrate metabolism. Such techniques are particularly needed for the study of the reactions involved in the formation of thromboplastic compounds in the blood. The observation that the lipid inhibitor of brain is ineffective in the presence of preformed convertin permits us to take a step in this direction. It allows us to distinguish quickly between clotting activity due to the simultaneous presence of tissue thromboplastin (or its equivalent in the blood, platelet-lipoid factor plus antihemophilic A factor plus antihemophilic B factor) and proconvertin from the activity produced when these compounds have reacted together to form convertin. Furthermore, it serves to emphasize that convertin is a qualitatively distinct activity not to be confused with the activity of proconvertin in either its plasma or serum form.

The inhibitor described by Overman and Wright in the inositol phosphatide fraction of brain, lung, plasma, and soybean had been thought to be identical to the lipid inhibitor of Tocantins.[18] Our findings clearly demonstrate that the two inhibitors act differently. Furthermore, since the inositol phosphatide inhibitor remains effective in the presence of convertin, it should not be called an antithromboplastin, at least not in the accepted meaning of the term thromboplastin. We have not been able to establish with certainty the mode of action of the soybean inositol phosphatide inhibitor, although our data are compatable with the hypothesis that it acts as an anticonvertin.

The lipid inhibitor of brain is quite different, as Lanchantin and Ware[19] have pointed out, from an inhibitor of thromboplastin which can be demonstrated upon the incubation of adsorbed serum with thromboplastin in the presence of calcium. The relation of the lipid inhibitor to the "prothrombin" inhibitor described by Fiala[20, 21] is less clear, although Fiala has stated that they are different.

SUMMARY

The lipid inhibitor described by Tocantins has been studied as it exists in the crude cephalin fraction of human brain. It has been found to block the reaction between tissue thromboplastin and proconvertin to form convertin, but to be completely ineffective in the presence of preformed convertin. It is useful, therefore, as a simple method to distinguish between proconvertin and convertin activity.

A sample of the soybean inositol phosphatide inhibitor described by Overman and Wright has been found to act differently from the lipid inhibitor for it remained fully active in the presence of preformed convertin. These two inhibitors had previously been thought to behave identically.

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COMPARISON OF INCINERATION AND CHLORIC ACID METHODS FOR DETERMINATION OF CHEMICAL PROTEIN-BOUND IODINE

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AS A result of the great value of the serum chemical protein-bound iodine determination in the evaluation of patients with hyperthyroidism and hypothyroidism, many attempts have been made to improve and simplify the procedure.[1] The most accurate and widely used methods involve isolation of the iodine by distillation before colorimetric determination. The distillation methods are very time-consuming, each sample requiring individual attention. Of the various attempts made to shorten the procedure, one of the best has been the alkaline-incineration method of Barker, Humphrey, and Soley[2] which does not require individual attention for each sample. Successful application of this method requires meticulous attention to details of technique and cleanliness. This, however, is unavoidable in any procedure attempting to measure minute concentrations of as ubiquitous a substance as iodine.

Recently, a chloric acid digest mixture has been employed for digestion and oxidation of the serum protein, leaving a soluble residue containing the iodine.[3, 4] Since the procedure appeared to be a simplification and improvement over other methods, it was compared with the incineration method. This report presents the results of this comparison.

METHODS AND MATERIALS

Chemical Procedures.—The alkaline-incineration method is essentially that described by Barker, Humphrey, and Soley.[2] We used an Evelyn colorimeter and made our readings at 4, 8, and 12 minutes instead of 6 and 12 minutes, as recommended. All determinations were made in duplicate. At no time have we had difficulty with contamination. This we attribute to the fact that glassware and equipment for this procedure are set up in a separate small room not utilized for any other purpose.

The chloric acid method is essentially O'Neal and Simms'[4] modification of the method of Zak and co-workers.[8] The O'Neal-Simms modification was elected because of the detailed information supplied regarding conditions affecting the determination, and the advantages of certain of their modifications. Determinations were made in duplicate, and an Evelyn colorimeter was used. With this, as well as the incineration method, recoveries of thyroxine were made with each set of determinations.

According to its advocates, the chloric acid method is much more rapid than previously described methods, including the incineration method. The latter requires two days for completion; however, much of this time consists of periods of waiting, during which other work can be done. In terms of actual technician-time spent on the procedures

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Received for publication, April 28, 1954.

we found little to choose between the two. The determination of twenty separate samples took approximately eight hours by the chloric acid method and nine hours by the incineration method.

Case Material.—Sera for these procedures were obtained from 14 patients who were myxedematous, 22 patients who were hyperthyroid, and 50 patients who were euthyroid. The diagnoses were unequivocal, being supported by simultaneous determinations of radioiodine uptake and clearance, protein-bound radioiodine concentration and conversion ratio determined at twenty-four hours, and basal metabolic rate and serum cholesterol concentration, in addition to the clinical findings.

Analysis.—Frequency distributions of the results obtained by each of the two methods in each group of patients were compared. Means and standard deviations (SD) of the individual test values as well as the differences between the methods were calculated. The mean differences were tested for significance by calculating "t" values and recording the probabilities that the mean differences observed were chance deviations from zero.

RESULTS AND INTERPRETATIONS

The frequency and cumulative frequency distributions of the values obtained with each group and each method are given in Fig. 1. The means and measures of variability corresponding to these distributions are listed in Table I. The ranges of variation of the two methods are similar in the hypothyroid and hyperthyroid groups. However, the values by the chloric acid method tend to be displaced upward slightly in the euthyroid range. The mean differences between the methods are 0.1, 1.3, and −0.4 μg per cent for the hypothyroid, euthyroid, and hyperthyroid groups, respectively. The mean differences observed for the two abnormal groups are insignificant; however, that for the euthyroid group is highly significant, as shown in Table II.

TABLE I. MEANS AND VARIABILITY OF VALUES OBTAINED WITH THE INCINERATION AND CHLORIC ACID METHODS, BY GROUP

	N	MEAN	SD	RANGE
Hypothyroid				
Incineration	14	1.5	0.8	0.5- 2.9
Chloric Acid	14	1.6	1.0	0.3- 3.8
Euthyroid				
Incineration	50	5.4	1.1	3.5- 7.9
Chloric Acid	50	6.7	1.0	4.6- 9.3
Hyperthyroid				
Incineration	22	15.4	5.0	8.2-29.4
Chloric Acid	22	15.0	4.7	8.8-27.7

TABLE II. TESTS OF SIGNIFICANCE OF MEAN DIFFERENCES BETWEEN METHODS, BY GROUP

	N	MEAN	SD	t	P
Hypothyroid	14	0.1	0.9	0.35	> 0.7
Euthyroid	50	1.3	1.2	7.68	$<10^{-9}$
Hyperthyroid	22	−0.4	2.1	−0.87	> 0.3

The mean of 6.7 μg per cent obtained by us with the chloric acid method on 50 euthyroid subjects is comparable to that of 7.3 μg per cent observed by Zak, Koen, and Boyle.[5] Their range of variation, 3.5 to 11.3 μg per cent, was greater, probably in part because their sample was larger (120 cases). The average value of 5.2 μg per cent reported by O'Neal and Simms was based upon ten cases only and cannot be considered representative.

From the cumulative frequency distributions of Fig. 1, it is clear that the entire distribution of euthyroid values obtained with the chloric acid method has been shifted upward by approximately 1 to 1.5 μg per cent. Since the hyperthyroid values are not shifted upward correspondingly by the method, the net result is slight loss in the degree of separation of the two groups.

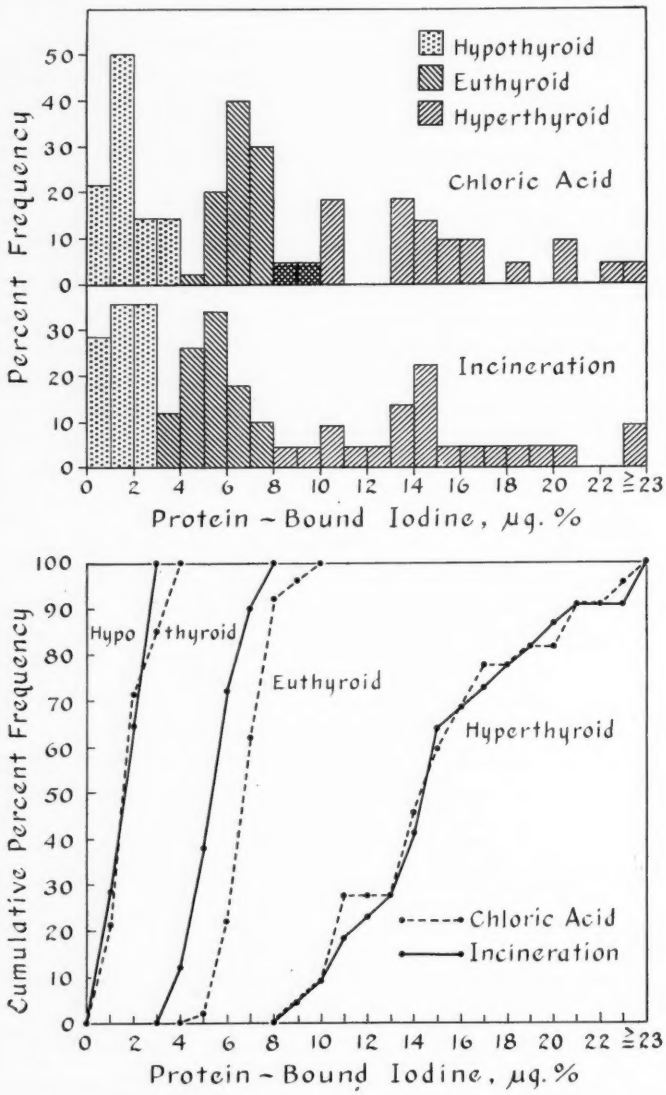

Fig. 1.—Frequency and cumulative frequency distributions of values obtained with the incineration and chloric acid methods, by group.

Whatever simplification gained with the chloric acid method is thus more than offset by loss in discriminative effectiveness in the zone of greatest interest to us, viz., 8 to 11 μg per cent.

SUMMARY

A comparison has been made between serum chemical protein-bound iodine values obtained with an incineration method and a chloric acid method in fourteen hypothyroid, fifty euthyroid, and twenty-two hyperthyroid patients.

There were no significant differences between the two methods in the hypothyroid and hyperthyroid groups. However, the entire distribution of euthyroid values obtained with the chloric acid method was shifted upward by approximately 1 to 1.5 μg per cent. Since the hyperthyroid values were not shifted upward correspondingly by this method, the net effect was loss in discriminative effectiveness of the test.

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HEPARIN AND THROMBOPLASTIN

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DURING the coagulation of blood in vitro an active thromboplastin is generated. The rate of formation of this thromboplastin determines, to a large extent, the clotting time of the blood. By injection of heparin into human subjects it is possible to prolong the whole blood clotting time to a considerable extent with little or no alteration in the one-stage prothrombin time. As the clotting time of whole blood is dependent on the evolution of *native* thromboplastin, and since an excess of thromboplastin is present in the measurement of the one-stage prothrombin time, *it seems probable* that the anticoagulant action of heparin is due, in part, to interference with thromboplastin.

In 1946 Jorpes[1] remarked that further consideration of this action of heparin was "hampered by the scantiness of our knowledge of thromboplastin." Recently Biggs, Douglas, and Macfarlane[2] have shown that a very active thromboplastin can be produced by incubating a mixture of platelets, antihemophilic factor, and serum accelerators in the presence of calcium. This is the basis of the thromboplastin generation test.[3] If one of the normal constituents in the mixture is replaced by material prepared from a patient under investigation, the thromboplastin produced can be compared with the yield when all constituents are normal. In this manner each component may be studied in turn.

It was in the light of this new knowledge of thromboplastin that the present investigation of the antithromboplastic action of heparin was undertaken. Three aspects of the problem were studied: (1) the effect of injected heparin on the clotting factors; (2) the effect of heparin on thromboplastin generation in vitro; (3) the effect of heparin on brain thromboplastin activity.

METHODS

Solutions of heparin were provided by the Connaught Laboratories, University of Toronto.

Brain thromboplastin: Difco rabbit brain thromboplastin was used.

Serum: Blood was allowed to clot in a glass tube at 37° C. After standing for twenty-four hours, the serum was separated and kept in the frozen state.

Clotting times were measured by a three-tube modification of the Lee and White method.

Prothrombin times of citrated plasma were determined by the one-stage method of Quick.

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This work was supported by a Grant from the National Research Council of Canada.
Received for publication, May 19, 1954.

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Thromboplastin generation test: In the performance of this test, washed platelets suspended in normal saline solution were used. Citrated plasma treated with $Al(OH)_3$ was used as a source of antihemophilic globulin and factor V. The $Al(OH)_3$ removes prothrombin, Christmas factor (which we consider to be the same as plasma thromboplastin component [PTC] of Aggeler[4]), and factor VII from the plasma. Normal serum prepared as described previously provided the serum accelerators, viz: factor VII and Christmas factor (PTC). These three components were incubated together in the presence of calcium, and samples of the mixture were tested at intervals for thromboplastin activity. Citrated plasma from normal subjects was centrifuged at 15,000 r.p.m. for ten minutes to remove platelets and was then employed as a substrate to test the thromboplastin activity of the incubation mixture. The shape of the curve showing thromboplastin generation varied slightly from day to day. To overcome this variability the results, using various test materials, were always compared with control values obtained on the same day.

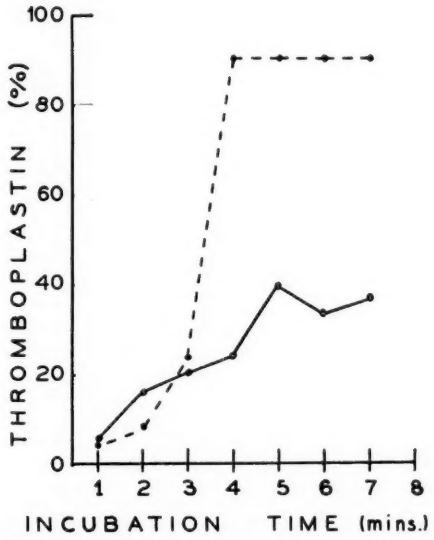

Fig. 1.—Thromboplastin generation in mixtures containing normal serum (----------) and serum from a patient given heparin (——————). In each instance the serum was diluted 1 : 10 with normal saline.

OBSERVATIONS

A. *Effect of Injected Heparin.*—Normal subjects were given various amounts of aqueous heparin by intravenous or intramuscular injection and samples of whole blood and citrated plasma were obtained at intervals by venipuncture. It was then possible to measure the effect of heparin on the various clotting components. The *prothrombin time* was not altered except when large amounts of heparin were given; the *clotting times* were longer than three times the normal value. Our studies were primarily concerned with doses of heparin of the magnitude employed in the treatment of thromboembolic disorders.

Washed *platelets* prepared from the blood of subjects receiving heparin behaved as normal platelets in the thromboplastin generation mixture. Similarly, when the plasma was treated with $Al(OH)_3$, thromboplastin formation was normal. This indicated that heparin did not destroy or permanently alter either *antihemophilic globulin* or *factor V*. It was interesting to note that when plasma

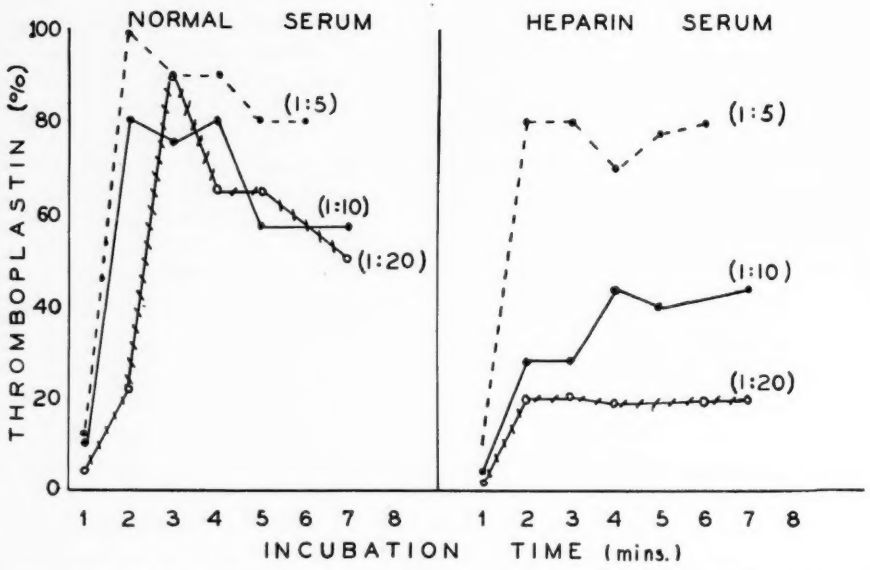

Fig. 2.—Thromboplastin formation using various dilutions of normal serum (*A*) and of serum from a patient receiving heparin (*B*).

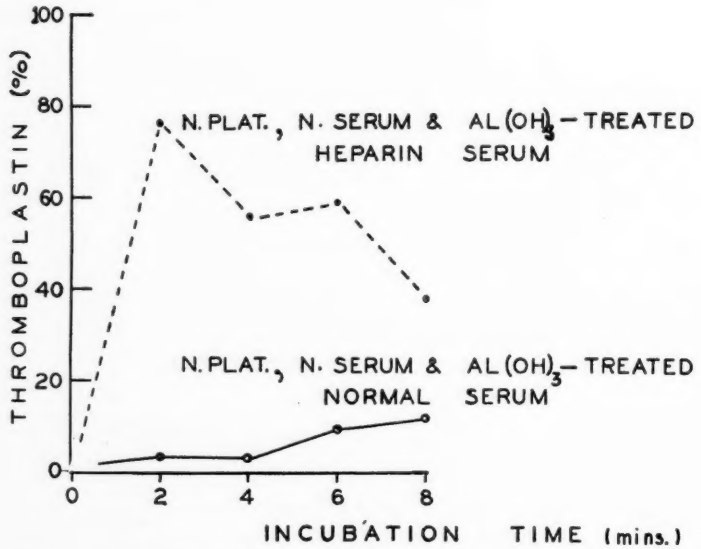

Fig. 3.—Thromboplastin generation curves demonstrating that serum from a patient receiving heparin contains antihemophilic factor.

TABLE I. CLOTTING FACTORS IN NORMAL SERUM AND IN "SERUM" FROM A PATIENT RECEIVING HEPARIN

	NORMAL SERUM	HEPARIN "SERUM"
Prothrombin	Trace	++
Serum accelerators	++	+
Antihemophilic factor	0	+

was treated with $Al(OH)_3$, heparin was absorbed from it. On the other hand, "serum" from a heparin-treated subject was found to be less effective than normal serum in the production of thromboplastin (Fig. 1). When higher concentrations of this serum were used, the yield of thromboplastin increased toward normal (Fig. 2). This suggested that the heparin serum was defective in accelerators. If the abnormality was simply due to heparin in the serum, increasing the concentration of serum would be expected to produce less thromboplastin.

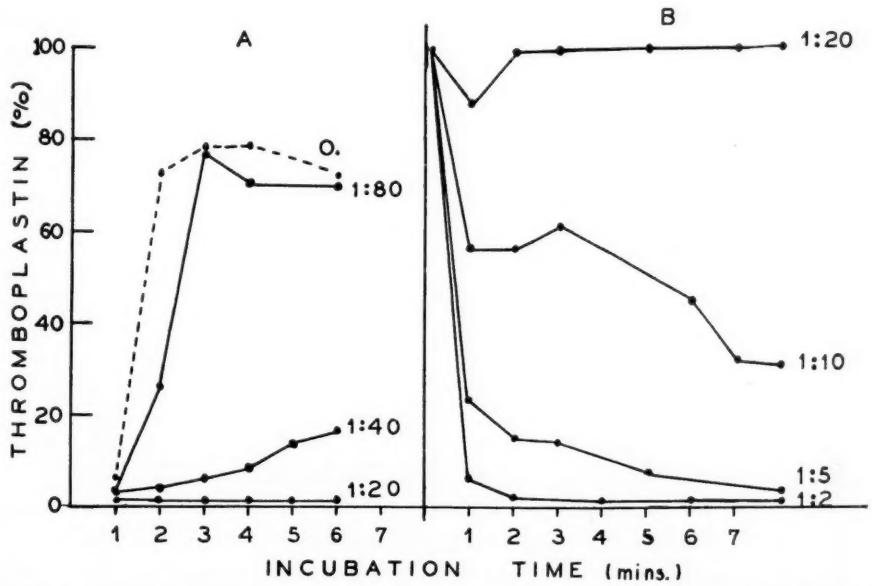

Fig. 4.—The effect of heparin added to the thromboplastin generation mixture: (*A*) prior to incubation, (*B*) after incubation had resulted in development of an active thromboplastin. The dilutions indicate the concentration of heparin in units per milliliter.

During the process of normal blood clotting, the antihemophilic factor disappears and normal serum does not contain this substance. It was present in the serum from heparin-treated subjects. To demonstrate this, heparin serum was treated with $Al(OH)_3$ to remove prothrombin, Christmas factor, and factor VII, and this was substituted for $Al(OH)_3$ normal plasma in the thromboplastin generation test. Under these circumstances a good yield of thromboplastin was obtained (Fig. 3). This indicated that clotting had been imperfect and the so-called heparin "serum" contained many plasma factors. In contrast to normal serum, heparin serum contained prothrombin, appreciable amounts of antihemophilic globulin, but small quantities of accelerators (Table I). After

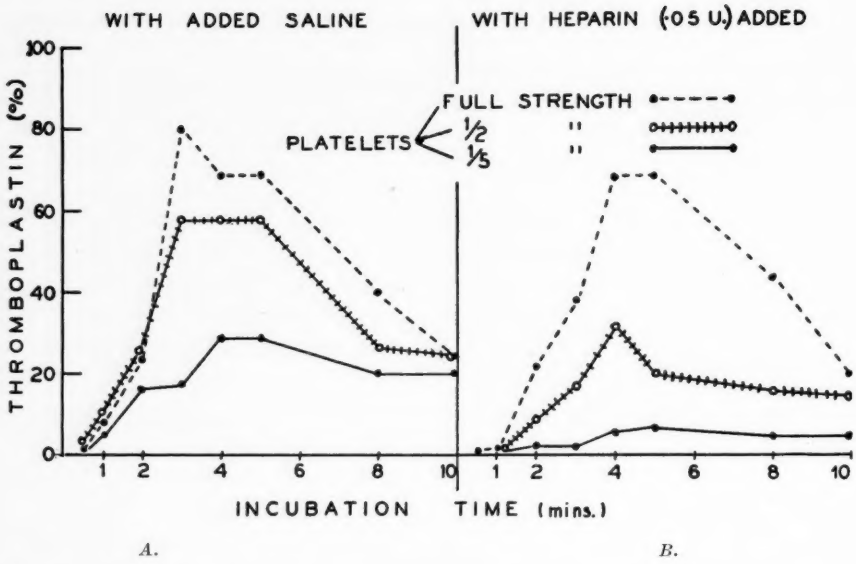

Fig. 5.—Curves demonstrating impaired thromboplastin generation with decreased concentrations of platelets (*A*), and the increased inhibitory effect of heparin in such mixtures (*B*).

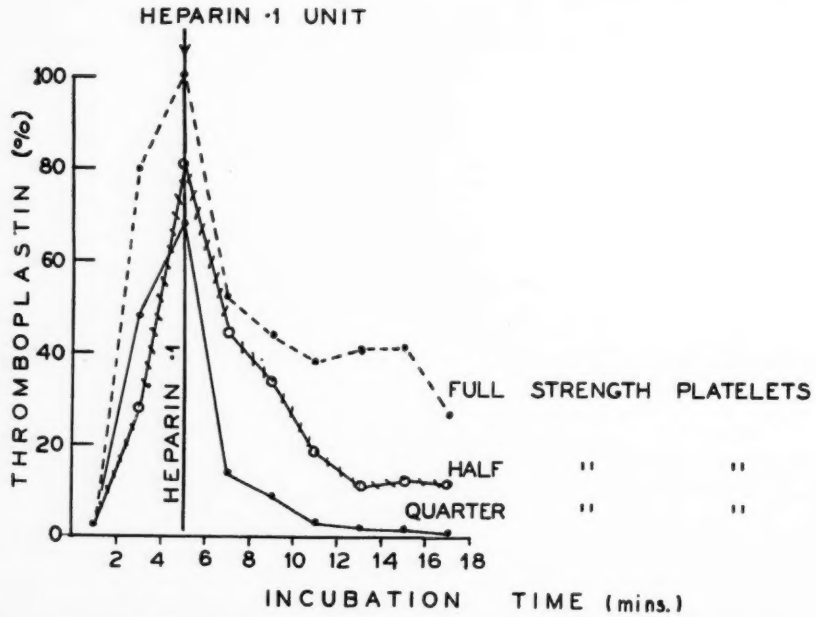

Fig. 6.—Curves demonstrating the influence of the platelet content on the destruction of preformed thromboplastin by heparin. In each case the thromboplastin generation mixtures, with platelet concentrations as shown, were incubated for five minutes before addition of the heparin.

heparin serum had been stored for a few days, its ability to accelerate thromboplastin formation increased and eventually it behaved as normal serum.

B. *Heparin and Thromboplastin General Mixtures.*—In the second part of the study it was found that the addition of heparin to the thromboplastin generation mixture delayed the rate of thromboplastin formation. With increasing

quantities of heparin, less thromboplastin was formed. For example, when the concentration of heparin in the mixture was one-eightieth unit per milliliter, delayed thromboplastin formation was observed; when the concentration was one-twentieth unit per milliliter, no thromboplastin was produced (Fig. 4, *A*). Heparin added to the generation mixture after thromboplastin had been formed led to destruction of thromboplastin although larger amounts of heparin were required. A concentration of one-twentieth unit of heparin per milliliter, which completely inhibited formation, did not destroy preformed thromboplastin prepared from a mixture of the same components (Fig. 4, *B*). These observations were in agreement with those of Biggs, Douglas, and Macfarlane.[5]

Under normal circumstances the yield of thromboplastin was reduced if the concentration of platelets was decreased. The inhibitory effect of a given amount of heparin was increased if the platelet concentration was reduced below normal levels in the thromboplastin generation mixture to which heparin was added (Fig. 5). Similarly, heparin induced more rapid and complete destruction of preformed thromboplastin prepared from mixtures containing smaller amounts of platelets (Fig. 6). These findings were in accord with Conley's[6] observation that platelet-poor plasma showed increased sensitivity to heparin.

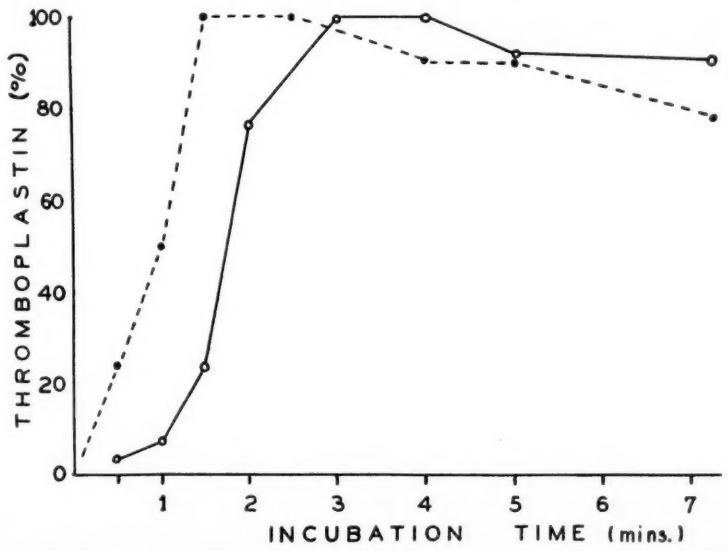

Fig. 7.—Curves demonstrating the accelerating effect of small amounts of active thromboplastin on the generation of thromboplastin (.-------.); 0.1 ml. of active thromboplastin was added prior to incubation. In the control mixture (o———o) no thromboplastin was added.

If one adds 0.1 ml. of active thromboplastin, which clots the substrate in ten seconds, to a normal thromboplastin generation mixture (total volume 1.3 ml.), marked acceleration of thromboplastin formation is observed. (See Fig. 7.) This effect is not due to direct action on the substrate since the small amount of thromboplastin when diluted with saline solution to the same volume (1.3 ml.) clotted the substrate in only fifty seconds (equivalent to a thromboplastin potency of 6 per cent of the normal). Active thromboplastin apparently acts as a catalyst accelerating the further production of thromboplastin. Similarly, the ad-

dition of 0.1 ml. of one-eightieth dilution of rabbit brain thromboplastin to a thromboplastin generation mixture caused acceleration of thromboplastin formation. On this basis the pronounced inhibitory effect of heparin on thromboplastin formation might be attributed to inactivation of thromboplastin as it was being formed. This would offset the autocatalytic effect of minute amounts of active thromboplastin and might explain why more heparin was required to destroy preformed thromboplastin.

Once thromboplastin had been destroyed by heparin the further addition of any of the three components (platelets, $Al(OH)_3$ plasma, or serum) to the mixture did not restore thromboplastic activity. It would appear that heparin interferes with the formation of thromboplastin and destroys formed thromboplastin but does not react with any single component required for thromboplastin generation.

C. *Heparin and Brain Thromboplastin.*—As a third approach to this problem, it was decided to study the effect of heparin on brain extract, which is an incomplete thromboplastin. The addition of normal serum to brain extract greatly increases its thromboplastic activity. Brain extract diluted 1:4 with saline solution was used to increase the sensitivity to heparin and platelet-poor citrated normal plasma was used as substrate. The results of the brain extract studies are summarized in Table II.

TABLE II. THROMBOPLASTIN MIXTURES CONTAINING RABBIT BRAIN. THROMBOPLASTIN WAS TESTED ON A SUBSTRATE OF NORMAL PLATELET-POOR PLASMA. CLOTTING TIME WAS RECORDED IN SECONDS

MIXTURE NUMBER	BRAIN 1:4 (ML.)	NaCl 0.85 (ML.)	$CaCl_2$ M/40 (ML.)	HEPARIN: 0.1 ML. = 1/10 UNIT (IN NaCl) (ML.)	NORMAL SERUM 1:10 (ML.)	DICUMAROL SERUM 1:10 (ML.)	CHRISTMAS SERUM 1:10 (ML.)	TIME OF INCUBATION (MINUTES) 1	3	5	7	9	11	
								(seconds)						CLOTTING TIMES
1	0.3	0.4	0.3					22	23	22				
2	0.3	0.3	0.3	0.1				21	22	22				
3	0.3	0.1	0.3		0.3			10	11	11	12			
4	0.3		0.3	0.1	0.3			11	15	16	19	21	26	
5	0.3	0.1	0.3			0.3		16	16	16				
6	0.3		0.3	0.1		0.3		18	19	18	21	22	21	
7	0.3	0.1	0.3				0.3	10	11	10				
8	0.3		0.3	0.1			0.3	11	15	15		16	17	
9	0.3	0.4			0.3			20	21	20				
10	0.3	0.3		0.1	0.3			21	21	23				

When normal serum was added to brain extract (mixture 3), the thromboplastic activity was rapidly increased and the clotting time decreased to one-half its original value. The addition of heparin to this mixture (mixture 4) resulted in a gradual deterioration over several minutes. Serum from a patient receiving Dicumarol (deficient in factor VII) did not enhance the activity of brain extract (mixture 5) as well as did normal serum or serum from a patient with Christmas disease (PTC deficiency) (mixture 7). Whenever improvement by serum occurred, the addition of heparin reduced the thrombo-

plastic activity. The same amount of heparin added to brain thromboplastin, without serum present, had no apparent effect even after incubation (mixture 2 compared to mxiture 1). It was also seen that calcium was necessary for the activation of brain by serum and for the effect of heparin (mixtures 9 and 10).

DISCUSSION

The concept that heparin affects the early stages of blood coagulation was introduced by Howell[7] in 1917. He found that a marked delay in the coagulation of blood caused by a moderate amount of heparin could be offset by addition of cephalin. This suggested that heparin acted against prothrombin and, for a year or so after its discovery, heparin was known as antiprothrombin. In 1938, Quick[8] concluded that heparin and thromboplastin had unrelated actions and that the activity of heparin could be explained by its antithrombin effect. On the other hand, Brinkhous and co-workers,[9] in 1939, showed that in the presence of plasma or serum heparin prevented the conversion of prothrombin to thrombin by thromboplastin. They postulated that heparin with an unidentified substance acted as an antiprothrombin or an antithromboplastin.

The apparent inconsistencies reported in the literature regarding the action of heparin probably arise from the many variables in the experimental procedures employed. Citrated blood behaves differently from oxalated blood. Various concentrations of heparin may affect the clotting mechanism differently and it seems that the antithrombic action is more pronounced with large amounts of heparin. Similarly, varying the concentration of thromboplastin or platelets or serum may change the results.

Since the formation of thromboplastin and the destruction of thromboplastin by heparin are time-consuming reactions, the length of time that the various components are in contact prior to the assay of the mixture is important. In our studies an attempt was made to limit these variables by employing concentrations of heparin which approximated those obtained clinically and by sampling the mixtures at definite intervals.

SUMMARY

Serum from patients receiving heparin was found to differ from normal serum in that it contained antihemophilic factor and prothrombin but smaller amounts of serum accelerators. These findings suggested that injected heparin interfered with blood clotting at a stage earlier than prothrombin conversion.

In experimental thromboplastin generation, heparin delayed the formation of thromboplastin and decreased the amount formed. Larger amounts of heparin would destroy preformed thromboplastin. Heparin appeared to act against active thromboplastin and not against any single thromboplastin component. If the thromboplastin was reduced by decreasing the platelet content, sensitivity to heparin was increased.

Small amounts of active thromboplastin were found to accelerate the further formation of thromboplastin, which suggested an autocatalytic reaction.

This might explain why larger amounts of heparin were needed to destroy preformed thromboplastin than to interfere with its formation.

Brain extract is an incomplete thromboplastin requiring serum containing factor VII for its activation. It was found that heparin in the concentrations used did not affect brain thromboplastin alone but in contact with activated brain extract it caused progressive destruction of thromboplastic activity.

It seemed clear from these studies that heparin, in the concentrations used, delayed coagulation by its antithromboplastin action. This antithromboplastic effect could explain the prolongation of whole blood clotting time with the relatively slight change in the one-stage prothrombin time observed clinically after heparin administration.

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STUDIES ON THE *C* ANTIBODY OF GROUP O SERUM WITH SPECIAL REFERENCE TO ITS ROLE IN HEMOLYTIC DISEASE OF THE NEWBORN

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IT IS now well established that erythroblastosis fetalis at times is due to an incompatibility between the A-B-O blood groups of mother and baby. This blood group system, although long known and considered simple when compared to that of the Rh-Hr system, is much more complicated than commonly believed.

A series of multiple allelic genes determine the A-B-O blood group system, but of these, for simplicity, we shall confine ourselves here to three, namely, *A*, *B*, and *O*, and we shall also disregard complexities such as the various subgroups of A and additional blood factors such as **O**, $\mathbf{B}_{\mathrm{I}}$, $\mathbf{B}_{\mathrm{II}}$, $\mathbf{B}_{\mathrm{III}}$, $\mathbf{F}_{\mathrm{A}}$, etc.[1] Each allelic gene determines a corresponding agglutinogen on the surface of the erythrocyte. Each agglutinogen, in turn, has certain serologic characteristics or attributes called blood factors. Most, if not all agglutinogens are characterized by multiple blood factors which are identifiable by appropriate diagnostic antisera. Thus, the *A* gene determines the A agglutinogen of group A and group AB blood and this agglutinogen is characterized not merely by the **A** factor but also by the **C** factor[2] (Table I). Similarly, the *B* gene determines the B agglutinogen of group B and group AB blood, and agglutinogen B is characterized not merely by the **B** factor but also by the **C** factor.

ANTI-**A**, ANTI-**B**, AND ANTI-**C**

The symbols **C** and anti-**C** were first used in connection with the A-B-O blood group system by Moss in 1910. These symbols were later also used in the British nomenclature for the Rh-Hr blood group system, and now the same symbol is being used for two blood factors of two different blood group systems. This is not without precedent; the symbol **S** is being used for the M-N-S system as well as to designate secretors of the A-B-O system. Since we have no desire to add to the confusion which already exists in this field, we have maintained the status quo by using the symbol **C** in its original sense in this, as well as in our previous paper on this subject. This brings into focus the need for concerted cooperative action to clarify this and other problems of nomenclature, however, not only for blood factors already known but also to establish a satisfactory method for naming those that may be discovered in the future.

Yet using the symbol **C** should cause the reader no difficulty because the British nomenclature makes use of ordinary type to designate the C factor of the Rh-Hr blood group system, whereas throughout this paper we will adhere to the convention of printing in boldface type the symbol for a blood factor as well as the symbol for the antibody specific for this single factor. Ordinary type will be used for the symbols for phenotypes and for agglutinogens, and for antisera specific for agglutinogens when the blood factors involved are not specific. Italics will be used for genes and genotypes.

To be read in abbreviated form at the Fifth International Congress of Blood Transfusion, Paris, France, September, 1954.
Received for publication, May 24, 1954.
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TABLE I. COMPOSITION OF THE FOUR BLOOD GROUPS (THEORY OF THREE PRINCIPAL BLOOD FACTORS AND ISOANTIBODIES)

BLOOD GROUPS	RED BLOOD CELLS		SERUM ISOANTIBODIES
	AGGLUTINOGENS	BLOOD FACTORS	
O	*	*	Anti-**A**, anti-**B** and anti-**C**
A	A	**A** and **C**	Anti-**B**
B	B	**B** and **C**	Anti-**A**
AB	A and B	**A, B,** and **C**	None

(From Wiener, A. S.: Ann. Eugen. **18**: 1, 1953.)
*Agglutinogen O and blood factor **O** have been omitted to simplify the chart.

Group A serum contains anti-**B**, group B serum contains anti-**A**, group AB serum contains neither, while group O serum contains both. Since agglutinogens A and B are both characterized by factor **C**, no anti-**C** is present in serum of group A, B, or AB. Group O erythrocytes are the only cells which do not contain the **C** factor and therefore group O serum is the only variety which can contain antibodies specific for factor **C** (Table I).

When group A serum is titrated against group B cells, the titer value obtained represents its anti-**B** content. Similarly, when group B serum is titrated against group A cells, the value obtained is its anti-**A** content. In both these cases, a single antibody is reacting with a single corresponding blood factor.* On the other hand, since group O serum contains anti-**C** as well as anti-**A** and anti-**B**, titration against group A cells measures the combined effect of the two antibodies, namely, anti-**A** and anti-**C**, reacting with two blood factors, **A** and **C**, of group A erythrocytes. Therefore, the titer for group A cells of *unabsorbed* group O serum is designated as the anti-A titer and not anti-**A** as for group B serum, since the latter refers to an antibody specific for the **A** factor alone. For the same reason, the titer of *unabsorbed* group O serum for B cells (containing **B** and **C** factors) will be designated anti-B titer and not as anti-**B**.

Group O serum with its three antibodies, anti-**A**, anti-**B**, and anti-**C** is comparable to serum from a type rh individual containing the three antibodies, anti-$\mathbf{Rh_o}$, anti-**rh′**, and anti-**rh″**. When serum from such a type rh individual is titrated against type Rh_1 cells or type Rh_2 cells, the result may be designated simply as its anti-Rh titer. However, if the titer of each individual Rh antibody is determined by testing against appropriate cells, type Rh_o, type rh′, and type rh″, respectively, the three titer values obtained should be designated with the symbols anti-$\mathbf{Rh_o}$, anti-**rh′**, and anti-**rh″**, all in boldface type.

If cells containing the **C** factor without either the **A** or **B** factor were available, one could directly determine the amount of anti-**C** present in the sera of group O individuals, and almost all of the problems and difficulties with regard to anti-**C** titrations would disappear. However, since such blood, if it exists at all, appears to have an incidence of approximately one in 60,000,[2c] the titer of the **C** antibody must be determined indirectly by subtraction. To determine the titer of anti-**C** in group O serum (Table II) one must first determine the values for anti-A and anti-B. Then the serum is absorbed with group A cells, and the absorbed serum again titrated against B cells. The latter result measures only the anti-**B** titer, because the group A cells will have absorbed both the **A** and **C** antibodies. The difference between anti-B and anti-**B** titers presumably represents the anti-**C** titer (Table II). In the reverse experiment, the group O serum is absorbed with B cells; this serves to remove anti-**C** and anti-**B**, so that titration of the absorbed serum measures the anti-**A** content of the group O serum. Again, the difference between the titers anti-A and anti-**A** presumably equals the anti-**C** titer.† Theoretically, the anti-**C** titer calculated from the result of the absorption with A cells should approximately equal the anti-**C** titer obtained when the serum is absorbed with B cells. Actually, this is not always the case, nor would one, on reflection, expect it to be. First, all titration results are subject to at least a 100 per cent error. This error is magnified in anti-**C** titrations, because

*Disregarding certain complexities mentioned at the beginning of this article.

†A mixture of equal parts of Group A and Group B sera does not show a reduction in anti-A or anti-B titer when absorbed by heterologous cells.

such titers are estimated by taking the difference between two titers. Second, group A cells and group B cells may differ in avidity for anti-**C**; indeed, it would be remarkable if the **C** factor of group A and group B cells were identical. Third, the three antibodies present in group O serum generally occur in different relative and absolute amounts. For example, the titer of anti-**C** could be high and that of both anti-**A** and anti-**B** low, or anti-**A** and anti-**B** both be of high titer and anti-**C** of low titer, etc. Thus, the main problem with serologic examinations for anti-**C** is not so much the demonstration of the presence of the antibody as its accurate titer estimation.

In a previous communication[3] we showed that in *homospecific pregnancies* the average titers of antibodies present in sera of group O mothers and reactive for group A and group B cells were four times as high as the titers of the corresponding antibodies in sera from mothers of group A and group B. This difference in titers we attributed to the presence of anti-**C** in group O serum. In addition, the ratio of titers of A and B antibodies in the sera of mothers and babies of compatible blood groups was one-fourth as high when the mother belonged to group O as when the mother's blood was group A or group B. Rosenfield[4] has also presented evidence that isoantibodies are demonstrable in cord serum more often when the mother belongs to group O than when she belongs to group A or group B. This difference in placental transfer of maternal antibodies we attributed to the presence of anti-**C** in group O serum exclusively. Thus, babies having group O mothers are apt to acquire by placental transfer about sixteen times as much isoantibodies as babies with mothers of group A or group B. These findings, we concluded, are adequate to account for the excess of group O mothers with babies having A-B-O hemolytic disease, a fact which has been pointed out by Rosenfield.[4]

TABLE II. ABSORPTION EXPERIMENTS DEMONSTRATING THE PRESENCE OF ANTI-**C** IN A GROUP O SERUM

GROUP O SERUM	SALINE AGGLUTINATION METHOD (TITER IN UNITS)			ACACIA METHOD (TITER IN UNITS)			ANTI-GLOBULIN METHOD (TITER IN UNITS)		
Unabsorbed	anti-A 128	anti-B 64		anti-A 128	anti-B 64		anti-A 128	anti-B 64	
Absorbed with A_1 cells	[0]*	anti-**B** 4	anti-**C**† 60	[1]	anti-**B** 8	anti-**C** 56	[0]	anti-**B** 4	anti-**C** 60
Absorbed with B cells	anti-**A** 8	[0]	anti-**C**† 120	anti-**A** 8	[0]	anti-**C** 120	anti-**A** 8	[0]	anti-**C** 120

*The titers enclosed in brackets are to be disregarded when calculating anti-**A**, anti-**B**, and anti-**C** titers. They merely indicate the completeness of absorption with homologous cells, and would be expected to be zero in every case were absorption always complete.

†Anti-**C** = anti-A - anti-**A** = anti-B - anti-**B**.

It was also shown by us that with heterospecific pregnancies in which the mothers were group O and their babies group A, not only was there very little, if any, anti-A in the cord serum, but also anti-B titer was below the expected value. These results can be explained most readily by postulating that a large proportion of the antibodies which babies acquire by passive transfer from group O mothers through the placenta is anti-**C**, and this as well as either anti-**A** or anti-**B** is absorbed by the babies' erythrocytes.

With these results in mind, the purpose of this paper is to extend our previous work on the role of factor **C** in A-B-O hemolytic disease, and to present additional evidence obtained by absorption experiments which estimate the amount of anti-**C** in the sera of group O mothers and in the cord sera of their infants.

MATERIALS AND METHODS

Clotted venous blood specimens were obtained from twenty-three group O mothers immediately after labor. At the time of delivery of each infant, a clotted specimen of cord blood was also obtained. In sixteen of the twenty-three cases both mother and baby

TABLE III. ANTI-C RESULTS OF SERA OF MOTHERS (GROUP O) AND CORD BLOODS OF THEIR BABIES (GROUP O)

CASE NUMBER	MOTHER OR BABY	TITER IN UNITS																			AVERAGE TITER IN UNITS	
		SALINE AGGLUTINATION METHOD							ACACIA METHOD							ANTI-GLOBULIN METHOD					SALINE AGGLUTINATION METHOD	ANTI-GLOBULIN METHOD
		BEFORE ABSORBING		ABSORBING CELLS					BEFORE ABSORBING		ABSORBING CELLS					BEFORE ABSORBING		ABSORBING CELLS				
				A_1			B				A_1			B				A_1			B			
		ANTI-		ANTI			ANTI		ANTI		ANTI			ANTI		ANTI		ANTI			ANTI			
		A_1	B	[A_1]*	B	C	A_1	[B]*	C	A_1	B	[A_1]*	B	C	A_1	[B]*	C	A_1	B	[A_1]*	B	C	A_1	[B]*	C	ANTI-C	ANTI-C†
1	M	8	16	[0]*	8	8	4	[0]	4	32	32	[1]*	16	16	16	[1]*	16	16	32	[0]*	8	8	6	16
	B	2	2	[0]	0	2	0	[0]	2	32	32	[1]	8	24	8	[0]	24	32	32	[0]	8	24	4	[0]	28	2	26
2	M	32	128	[0]	16	112	6	[1]	26	48	160	[1]	20	140	10	[2]	38	40	160	[0]	64	96	8	[6]	32	69	64
	B	1	2	[0]	1	1	0	[0]	1	8	16	[1]	10	6	4	[0]	4	4	18	[0]	12	6	4	[1]	0	1	3
3	M	128	64	[0]	4	60	8	[0]	120	128	64	[1]	8	56	8	[0]	120	128	64	[0]	4	60	8	[0]	120	90	90
	B	4	4	[0]	0	4	0	[0]	4	64	24	[1]	8	16	4	[0]	60	64	64	[0]	8	56	4	[0]	60	4	58
4	M	64	64	[0]	8	56	8	[0]	56	128	128	[1]	16	112	16	[0]	112	64	64	[0]	8	56	16	[0]	48	56	52
	B	2	2	[0]	0	2	1	[0]	1	16	8	[1]	1	7	8	[0]	8	32	16	[0]	0	16	8	[0]	24	1½	20
5	M	64	64	[0]	8	56	16	[0]	48	128	128	[2]	8	120	32	[2]	96	128	128	[1]	8	120	64	[1]	64	52	92
	B	8	8	[0]	1	7	4	[0]	4	128	128	[8]	12	116	32	[4]	96	128	128	[1]	8	120	64	[2]	64	5½	92
6	M	128	64	[0]	32	32	40	[1]	88	256	128	[8]	48	80	64	[16]	192	128	64	[0]	32	32	40	[0]	88	60	60
		3	2	[0]	0	2	1	[0]	2	64	42	[8]	32	10	42	[6]	22	64	48	[1]	3	45	4	[1]	60	2	52
7	M	128	128	[1]	64	64	64	[0]	64	256	256	[8]	64	192	128	[4]	128	256	128	[2]	64	64	128	[0]	128	64	96
	B	8	2	[0]	0	2	2	[0]	6	128	32	[4]	8	24	32	[4]	96	128	32	[1]	8	24	32	[0]	96	4	60
8	M	32	16	[0]	16	0	16	[0]	16	64	32	[2]	16	16	16	[1]	48	64	32	[1]	16	16	32	[0]	32	8	24
	B	0	0	[0]	0	0	0	[0]	0	16	10	[2]	8	2	16	[2]	0	16	6	[1]	4	2	8	[1]	8	0	5
9	M	32	32	[0]	16	16	16	[0]	16	64	64	[2]	16	48	32	[1]	32	64	64	[0]	16	48	32	[0]	32	16	40
	B	2	2	[0]	1	1	1	[0]	1	32	16	[2]	16	0	16	[1]	16	64	32	[0]	32	0	32	[0]	32	1	16
10	M	64	32	[0]	8	24	32	[0]	32	128	32	[2]	8	24	32	[2]	96	128	32	[0]	8	24	64	[0]	64	28	44
	B	2	0	[0]	0	0	1	[0]	1	64	8	[2]	8	0	32	[2]	32	64	2	[0]	2	0	32	[0]	32	1½	16
11	M	64	32	[0]	16	16	32	[0]	32	128	64	[2]	16	48	32	[1]	96	128	128	[2]	32	96	128	[1]	0	24	48
	B	4	4	[0]	1	3	2	[0]	2	64	64	[2]	16	48	32	[4]	32	128	128	[1]	16	112	64	[1]	64	2½	88
12	M	64	64	[0]	32	32	32	[0]	32	128	64	[4]	32	32	32	[4]	96	128	64	[0]	64	0	32	[0]	96	32	48
	B	4	2	[0]	0	2	2	[0]	2	64	32	[4]	8	24	32	[4]	32	64	32	[0]	4	28	32	[0]	32	2	30
13	M	64	64	[1]	32	32	32	[0]	32	512	256	[8]	32	224	128	[4]	384	512	128	[2]	32	96	512	[0]	0	32	48
	B	4	1	[0]	1	0	2	[0]	2	256	32	[8]	8	24	128	[4]	128	256	16	[1]	4	12	256	[1]	0	1	6
14	M	64	64	[1]	64	0	64	[0]	0	64	64	[8]	64	0	64	[4]	0	64	64	[1]	64	0	64	[1]	0	0	0
	B	1	1	[0]	1	0	1	[0]	0	32	16	[4]	8	8	16	[4]	16	16	16	[0]	4	12	8	[0]	8	0	10
15	M	16	32	[0]	32	0	8	[1]	8	64	64	[1]	32	32	16	[1]	48	16	32	[0]	32	0	8	[1]	8	4	4
	B	2	4	[0]	2	2	1	[0]	1	32	32	[0]	2	30	16	[0]	16	4	4	[0]	4	0	4	[0]	0	1½	0
16	M	256	8	[4]	8	0	256	[1]	0	1024	32	[16]	8	24	1024	[8]	0	2048	32	[4]	8	24	2048	[2]	0	0	0
	B	32	1	[1]	1	0	32	[0]	0	512	16	[8]	4	12	512	[4]	0	1024	4	[2]	2	0	1024	[0]	0	0	0

M = Mother's serum.

B = Baby's cord serum.

*Titers enclosed in brackets are to be disregarded when calculating anti-A, anti-B, and anti-C titers. They merely indicate the completeness of absorption with homologous cells, and would be expected to be zero in every case were absorption always complete.

†The anti-C values listed here were obtained by averaging the two titers obtained by absorption with A_1 and B cells.

were group O. Among the remaining seven cases, although these mothers were also group O, six of the babies were group A and one was group B. The serum of each mother and each baby was divided into three parts. One part was retained unabsorbed, the second was absorbed with group A_1 cells, and the third was absorbed with group B cells. The cells used for absorption were washed three times with normal saline solution and then packed. One volume of serum was mixed with one volume of appropriate washed packed cells and placed in a water bath at 37° C. for one-half hour, then allowed to stand at room temperature for one-half hour, then centrifuged, and the serum removed. The six samples of unabsorbed and absorbed sera from each mother and her baby were simultaneously titrated, using identical techniques and group A_1 and group B test cells, by the saline agglutination, the acacia conglutination, and the antihuman globulin methods, thus yielding titers anti-A, anti-B, anti-**A**, and anti-**B**, from which the anti-**C** titer was calculated by simple subtraction. Table II has been prepared to illustrate the method of calculation and the formula.

Saline Agglutination Method.—Group A_1 cells, group B cells, and group O cells were used for these tests. The cells were fresh, washed once in normal saline solution, and of each variety a 2 per cent saline suspension in terms of cell sediment was prepared. Two rows (one for group A_1 cells and one for group B cells) of 10 small test tubes (7 to 8 mm. inside diameter) were placed in a rack. Two additional tubes were used for the group O control cells. A series of ten progressively doubled saline dilutions of the mother's serum was made; that is, undiluted, 1:2, 1:4, 1:8, etc., up to 1:512. In making these dilutions, in order to avoid carry-over of sera from a higher concentration to a lower concentration, the pipette was thoroughly rinsed in *fresh* saline solution after each dilution. Into the first tube of each row was placed 1 drop of the mother's undiluted serum. Into the second tube of each row was placed 1 drop of the 1:2 saline dilution. This was repeated for each successive tube using the next higher dilution so that in the tenth tube 1 drop of the 1:512 dilution was placed. To each of the ten tubes in the first row was added 1 drop of the group A_1 cell suspension. Into each of the ten tubes in the second row was added 1 drop of the group B cell suspension. Into each of the two control tubes was placed 1 drop of the group O cells. The tubes were shaken, placed in a water bath at 37° C. for one hour and the reaction read in the usual fashion under the microscope using the scanning lens. The last tube showing a definite (one-plus) reaction was the end point, and the titer was expressed in units equal to the reciprocal of the serum dilution in that tube.

Acacia Method.—The technique for this method was identical with that of the saline agglutination method, except that after both the saline dilutions of serum as well as the cells had been placed in the tubes, one drop of 10 per cent acacia mixture* was added. The tubes were shaken, placed in the water bath at 37° C. for one hour and then examined for clumping, as for the saline agglutination method, under the microscope using the scanning lens. Again, the titer was expressed in units equal to the reciprocal of the serum dilution in the last tube giving a definite (one-plus) reaction.

Antihuman Globulin Method.—This method[5] was merely a continuation of the saline agglutination method. The cell sediment in each tube in which no clumping was observed was washed three times with large volumes of normal saline solution and then packed. This was to remove all plasma, which contains globulin, and which would react with the antihuman globulin serum and produce a false negative result. The saline was removed as completely as possible. To the packed cells 2 drops of antihuman globulin serum (rabbit) was added, mixed, and centrifuged lightly (500 to 1,000 r.p.m.) for one minute. The sediment was gently resuspended, and examined for clumping under the microscope using the scanning lens. The tube with the highest serum dilution showing a definite (one-plus) reaction was considered the end point and the titer was expressed in units equal to the reciprocal of the dilution of the serum present in that tube.

*Ten grams of gum acacia and one gram of dibasic sodium phosphate (Na_2HPO_4) dissolved in 90 ml. distilled water and sterilized at once by autoclaving at 10 pounds pressure for 10 minutes.

†The anti-C values listed here were obtained by averaging the two titers obtained by absorption with A_1 and B cells.

RESULTS

Table III gives titration results of sera of 16 group O mothers and their group O babies. For each mother and her baby the anti-A and anti-B titers were obtained by the agglutination, acacia, and antiglobulin methods of titration, using the unabsorbed serum. After absorption with A_1 cells, the value for anti-**B** was obtained, which was subtracted from the value for anti-B, thus giving the value for anti-**C**. Similarly, after absorption with B cells the value for anti-**A** was obtained, and this was subtracted from the value for anti-A, thus yielding a second estimate of the anti-**C** titer.

Since, as will be shown later, anti-**C** is an antibody largely of the placenta-passing or univalent variety, the results obtained by the acacia and antiglobulin methods are the most important. Although titers by the former method are somewhat higher, the drawback of the acacia method is that it tends to produce a certain amount of stickiness and nonspecific clumping, as shown by the tests with the control cells. The antiglobulin method, which detects the identical variety of antibodies, has the advantage of greater reproducibility, so that throughout this paper the latter will be referred to by preference.

*Anti-**C** in the Mother's Serum.*—For each mother's serum the two calculated anti-**C** titers by the saline agglutination method showed reasonable agreement (Table III). When in each case the two values were averaged, the results showed that anti-**C** was also present in bivalent form in the serum of each mother except Cases 14 and 16, Table III. The average titer for the entire group was 35 units. These findings are in sharp contrast, as will be seen later, to the findings by this method in the sera of the babies.

In the first ten cases (Table III) not only was anti-**C** found in each *mother's* serum, but the two calculated titers (antiglobulin method) showed reasonable agreement. On the other hand, in Cases 11, 12, 13, 15, and 16, the anti-**C** titers calculated from the absorption with A_1 cells was different from the values obtained from the absorption with group B cells, because here one of each pair of calculated values was zero. However, discrepancies such as in Cases 11, 12, 13, and 15, could possibly be explained by postulating a one tube error in one or more of the antiglobulin titrations which is, admittedly, within the limits of error of the test. For example, in Case 12, if the anti-B titer by the antiglobulin method had been found to be 128 units instead of 64, then the anti-**C** titer, calculated from the results of absorption with A cells would have been 64 units, closely approximating the titer of 96 units calculated from the results of absorption with B cells. In Case 14, absorption with A cells did not reduce the antiglobulin titer for B cells at all; nor did absorption with B cells reduce the titer for A cells. This would indicate that the anti-**C** antiglobulin titer in this case was zero or low, and that the values obtained for anti-A and anti-B were actually those for anti-**A** and anti-**B**. Of course, as previously stated, differences in avidity of group A and group B cells for anti-**C** may also play a role. Thus, the last five cases highlight some of the problems of interpretation of results, and the difficulties in obtaining exact anti-**C** titers.

In order to understand these results better, let us consider some hypothetical cases, as illustrated in Table IV, in which all titration results are assumed to be perfect, so that human errors and the limitations of the test have been entirely eliminated. Let us assume that in the serum of a group O individual the anti-**C** titer is 100 units, the anti-**A** titer is 5 units, and the anti-**B** titer 5 units. Anti-A (anti-**A** plus anti-**C**) would then be 105 units and anti-B (anti-**B** plus anti-**C**) 105 units. The titration and absorption results in this case should then be represented in Table IV, Case 1. Such a combination of titers could account for actual cases in which there is a relatively large amount of anti-**C** while the titers for anti-**A** and anti-**B** are low; for example Table III, Case 3. In such a combination, namely, when anti-**C** is high and anti-**A** and anti-**B** are both low, the amount of anti-**C** present in a serum can be most easily and reliably determined. Let us assume, on the other hand, that the serum of another hypothetical group O individual also contains 100 units of anti-**C** but that the anti-**A** titer is high, namely 100 units, and the anti-**B** titer low, namely 5 units. The anti-A titer should then be 200 units and the anti-B titer 105 units (Table IV, Case 2). The two absorption results should show anti-**C** to be 100 units but since the difference between the anti-A and anti-**A** titers is only one serum dilution, the results obtained by absorption with B cells could appear equivocal. Such a combination of titers could account for some of the cases encountered in the present study (cf. Tables III, Cases 5, 10, and 13).

TABLE IV. ANTI-**C** RESULTS OF HYPOTHETICAL CASES

CASE NUMBER	TITER IN UNITS							
	BEFORE ABSORBING		ABSORBING CELLS					
			A_1			B		
	ANTI		ANTI			ANTI		
	A_1	B	[A_1]	**B**	**C**	A_1	[B]	**C**
1	105	105	[0]	5	100	5	[0]	100
2	200	105	[0]	5	100	100	[0]	100
3	200	200	[0]	100	100	100	[0]	100
4	105	105	[0]	100	5	100	[0]	5

Let us assume still another hypothetical group O individual (Table IV, Case 3) in whose serum the anti-**C**, the anti-**A**, and the anti-**B** are all relatively high, namely 100 units. The anti-A titer of the unabsorbed serum should therefore be 200 units and the anti-B titer also 200 units. Anti-**C** titers calculated from the absorption tests should then be 100 units, but since there would be a titer difference of only one tube in both absorption tests, the results could well be equivocal. Such a combination of titers could explain the results obtained in actual cases (cf. Table III, Cases 7, 8, and 9).

Instead of assuming, as in the previous hypothetical cases, that the anti-**C** titer is high, let us assume it is low, for example, 5 units, and that both the anti-**A** and anti-**B** titers are high, namely 100 units (Table IV, Case 4). In this case then, the anti-A titer should be 105 units, and the anti-B titer also 105 units. In actual practice, of course, it is impossible to distinguish a titer of 105 units from one of 100 units, and the findings could therefore lead one to

conclude that no anti-**C** is present (cf. Table III, Case 14). Still other situations could be hypothesized, but those given in Table IV are sufficient to highlight the problem.

*Anti-**C** in Baby's Serum.*—Our next objective was to apply the same titration and absorption methods to determine the amount of anti-**C** which had passed across the placental barrier and was present in the cord sera of the 16 group O infants born of group O mothers (Table III).

Since bivalent antibodies are demonstrable by the saline agglutination method, and since such antibodies are believed not to have the ability to traverse the placental barrier, one would expect to find no saline active antibodies in cord serum.[6] Indeed, with but a single exception (Case 16) there was little or no clumping of cells in saline media by cord serum, the titers ranging between zero and 8 units. This, as has been previously noted, is in sharp contrast to the finding of considerable amounts of bivalent antibodies in sera of mothers, with the titers ranging (again, with the exception of Case 16) from 8 to 128 units. In fact, the average titer by the saline agglutination method in cord serum was two units, as compared to an average titer by the same method of 35 units in mothers' sera. The low titers in sera of babies, obtained in saline media, we regard as artifacts in the sense that they actually are not the result of agglutination by bivalent antibodies, but the result of conglutination by univalent antibodies.[7] In the first or second tube of a saline agglutination titration, the concentration of protein of the cord serum under test may still be sufficiently high to produce some clumping in the presence of specific univalent antibodies (conglutination). As a matter of fact, in each case both the acacia and antiglobulin titrations confirmed the presence of considerable amounts of univalent antibodies. This concept is confirmed by the finding that as babies get older the titers of the A and B antibodies in their sera as determined by the acacia method, are progressively reduced by one-half at intervals of approximately thirty to forty days,[8C] which is the half life of antibodies,[8A, 8B] while the low titer obtained in saline media shows no corresponding reduction, confirming the idea that the latter reaction is due to conglutination.

Confining our attention to the average titers obtained by the antiglobulin method, it will be noted (Table III) that in general there is a close relationship between the anti-**C** titer of each mother's serum and the anti-**C** titer of the cord serum of her baby, which is the expected finding with univalent antibodies. The average titer (antiglobulin method) for the entire group of mothers' sera was 45 units and that for the entire group of cord sera, 30 units. Individually, when the anti-**C** titer of a mother's serum was high, moderately high, or low, there was usually a corresponding amount of anti-**C** in her baby's serum. Nevertheless, in Table III, Cases 2, 8, and 13 constitute exceptions to this rule in that the titer for anti-**C** in cord serum was low, even though the titer of the maternal serum was relatively high. Thus, the ratio between the mother's anti-**C** titer and that of her infant varied widely, but in most cases it was low; the average ratio being 1.7, indicating that when univalent anti-**C**

is present in the mother's serum, as a rule it readily passes across the placental barrier. In the three cases mentioned previously, the ratio was high, indicating that in these cases anti-**C** had failed to pass through the placenta in any considerable amount.

The fact that anti-**C** is an antibody which, in almost all cases, readily passes the placental barrier would not support the theory of Dodd[9] and Bird[10] that the antibodies found in group O serum which cross react with both group A and group B cells are molecules of anti-**A** and anti-**B** linked together. We have shown that relatively little anti-**A** or anti-**B** traverses the placenta from mothers of group B and group A, respectively. It would seem that linking these antibodies together would make passage through the placental barrier even more difficult. Yet our observations show that the antibody in the serum of group O mothers, which cross reacts with both group A and group B cells, is of the variety that readily traverses the placental barrier, and this supports our view that it is a separate antibody of low molecular weight, namely, anti-**C**.

Varieties of **C** *Antibodies.*—A number of heterospecific pregnancies were also studied, namely, a group of cases consisting of seven group O mothers, six of whom had group A babies and one a group B baby (Table V). To the sera of these mothers and babies the same methods of titration were applied, before and after absorption with A_1 and B cells.

Since the explanation of the results is identical for all seven cases, a detailed description of any one, for example, Case 1, Table V, will suffice. The three methods of titration showed, as was to be expected, that only univalent antibodies had traversed the placenta. The cord serum had an anti-A titer of 2 units by the saline agglutination method, but as has already been pointed out, we consider this type of reaction an artifact due to conglutination. Since the mother belonged to group O, anti-**A**, anti-**B**, and anti-**C** present in her serum could be expected to pass into the cord serum of her infant. However, since the baby belonged to group B, one would expect the infant's cells to absorb any passively acquired anti-**B** and anti-**C**, leaving only anti-**A** free in the cord serum. In fact, the baby's serum contained 128 units (antiglobulin method) of isoantibodies reactive for group A_1 cells, which one could assume to be anti-**A** and there was no anti-**B**. When this serum was absorbed with group A_1 cells, the serum lost its ability to agglutinate group A as well as group B cells, as was to be expected. However, when the serum was absorbed with an excess of group B cells, the titer of the baby's serum for group A cells was reduced from 128 units to 8 units, which at first sight seems contrary to expectation. This result could be explained by postulating that the antibody detected in the cord serum was actually not anti-**A**, but a form of anti-**C** with a greater avidity for agglutinogen A than for agglutinogen B (cf. Fig. 1). Similar seemingly paradoxical findings have been obtained with sera of specificities anti-A_1 and anti-N. For example: anti-A_1 serum might have a titer of 8 units for A_1 cells and zero for A_2 cells, yet absorption with an excess of group A_2 cells will reduce the titer for A_1 to zero.

TABLE V. ANTI-C RESULTS OF SERA OF MOTHERS AND CORD BLOODS OF THEIR BABIES
(HETEROSPECIFIC PREGNANCIES)

CASE NUMBER	MOTHER OR BABY	TITER IN UNITS																			
		SALINE AGGLUTINATION METHOD								ACACIA METHOD								ANTIGLOBULIN METHOD							
		BEFORE ABSORPTION		ABSORBING CELLS						BEFORE ABSORPTION		ABSORBING CELLS						BEFORE ABSORPTION		ABSORBING CELLS					
				A_1			B					A_1			B					A_1			B		
		ANTI		ANTI			ANTI			ANTI		ANTI			ANTI			ANTI		ANTI			ANTI		
		A_1	B	$[A_1]$‡	B	C	A_1	$[B]$‡	C	A_1	B	$[A_1]$‡	B	C	A_1	$[B]$‡	C	A_1	B	$[A_1]$‡	B	C	A_1	$[B]$‡	C
1*	M†	32	32	[0]	16	16	32	[0]	0	128	32	[4]	32	0	64	[4]	64	256	32	[0]	16	16	128	[0]	128
	B†	2	0	[0]	0	0	0	[0]	2	64	4	[2]	1	3	16	[0]	38	128	0	[0]	0	0	8	[0]	120
2	M	32	32	[0]	16	16	32	[0]	0	64	32	[2]	32	0	14	[1]	50	32	32	[0]	16	16	32	[0]	0
	B	0	2	[0]	0	2	0	[0]	0	2	32	[0]	8	24	2	[20]	0	0	16	[0]	4	12	0	[0]	0
3	M	8	8	[0]	4	4	4	[0]	4	16	16	[0]	8	8	8	[0]	8	16	8	[0]	4	4	8	[0]	8
	B	0	0	[0]	0	0	0	[0]	0	0	8	[0]	4	4	0	[0]	0	0	4	[0]	1	3	0	[0]	0
4	M	32	32	[0]	16	16	16	[0]	16	64	64	[0]	16	48	16	[0]	48	32	32	[0]	16	16	16	[0]	16
	B	0	1	[0]	0	1	0	[0]	0	0	8	[0]	4	4	0	[0]	0	0	4	[0]	0	4	0	[0]	0
5	M	16	32	[0]	16	16	16	[0]	0	32	32	[0]	16	16	16	[0]	16	32	32	[0]	16	16	16	[0]	16
	B	0	0	[0]	0	0	0	[0]	0	0	8	[0]	4	4	0	[0]	0	0	4	[0]	1	3	0	[0]	0
6	M	4	4	[0]	4	0	2	[0]	2	16	16	[0]	16	0	8	[0]	8	4	4	[0]	4	0	2	[0]	2
	B	0	0	[0]	0	0	0	[0]	0	0	8	[0]	4	4	0	[0]	0	0	4	[0]	2	2	0	[0]	0
7	M	32	32	[0]	32	0	32	[0]	0	64	64	[2]	32	32	64	[0]	0	64	64	[0]	32	32	64	[0]	0
	B	1	1	[0]	1	0	0	[0]	1	16	16	[2]	16	0	16	[1]	0	0	16	[0]	8	8	0	[0]	0

*Mother group O; baby group B. In the remaining cases the mother was Group O but the baby in Cases 2, 3, and 4 was group A_1, and in Cases 5, 6, and 7, was group A_2.

†M = Mother's serum; B = her baby's cord blood.

‡Titers enclosed in brackets are to be disregarded when calculating anti-A, anti-B and anti-C titers. They merely indicate the completeness of absorption with homologous cells, and would be expected to be zero in every case were absorption always complete.

Studies on the cross reactions of anti-B agglutinins of human group A and group O sera show that these antibodies are not composed of a single uniform molecular type, but consist instead of a "spectrum" of antibodies with differing cross reactions. An excellent analysis of the cross reactions of anti-B of normal human serum and immune rabbit serum has recently been made by Owen.[11] Similarly, **C** antibodies are probably not all identical molecules, but a spectrum of closely related antibodies sharing the property of cross-reacting to greater or less degree with agglutinogen A and agglutinogen B. While probably most **C** antibodies react with equal avidity on A and B cells (anti-**C**), it is easy to conceive that some fractions of **C** antibodies might act with greater avidity on A than on B (anti-$\mathbf{C}_A$) while still other fractions might act with greater avidity on B than on A_1 (anti-$\mathbf{C}_B$) as indicated in the diagram (Fig. 1).

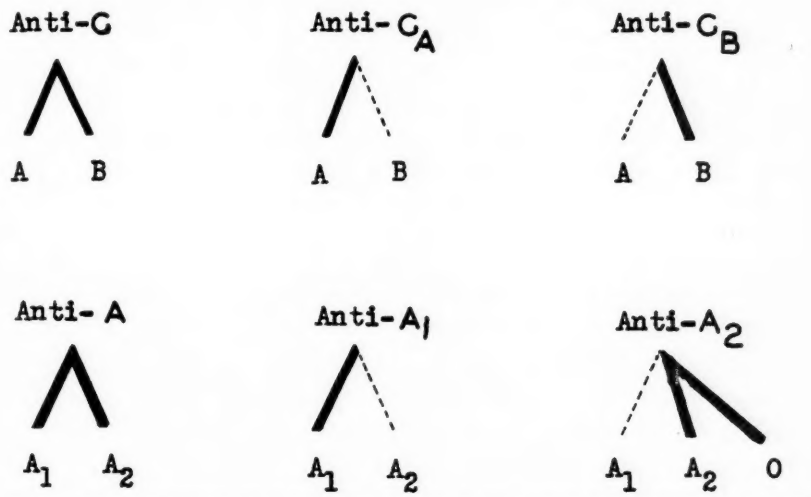

Fig. 1.—Hypothetical reactions of the "spectrum" of C antibodies compared with A antibodies. (The heaviness of the line indicates the avidity of the combination.)

CONCLUSIONS

Inasmuch as hemagglutinogens of group A, group B, and group AB individuals are each characterized by the **C** factor, the sera of such individuals do not contain anti-**C**. Erythrocytes of group O individuals lack the **C** factor as well as the **A** factor and the **B** factor, and the sera of such individuals do contain anti-**C**.

In a previous communication it was shown that babies having group O mothers are apt to acquire by placental transfer about sixteen times as much isoantibodies reactive for group A and group B cells as babies of either group A or group B mothers. It was concluded that a large proportion of the antibodies which the baby acquired by placental transfer from the group O mother was of specificity anti-**C**. The capacity, possessed only by group O mothers, to form anti-**C**, it was also concluded, accounts for the excess of group O mothers among babies having hemolytic disease.

This explanation is strengthened by the results of the absorption studies presented in this communication. When serum of a group O mother is absorbed with group A_1 cells, not only is the ability of that serum to react with group A_1 cells removed, but also the titer of that serum for group B cells is often reduced. This reduction in heterologous titers is due to absorption by A_1 cells of anti-**C** as well as anti-**A**, leaving only anti-**B**. Analogous results are obtained when sera of group O mothers are absorbed with group B cells; that is, the titer of the serum for group A cells is reduced by the amount of anti-**C** absorbed by group B cells. Thus, the anti-**C** titer can be determined by two methods, represented by the relationship, anti-**C** = anti-A – anti-**A** = anti-B – anti-**B**.

The anti-**C** content both of sera of group O mothers and cord sera of their babies was determined by the saline agglutination, acacia, and antiglobulin techniques. In cord serum, anti-**C** of the saline agglutinating variety was not detectable, even when it was found in considerable concentration in the serum of its mother. However, in most instances the titer for univalent anti-**C** in the infant's cord serum was comparable to the univalent anti-**C** titer of its mother's serum, indicating that the **C** antibody in the group O mother's serum is mostly of the placenta passing variety.

Antibodies in the sera of group B mothers and reactive for A cells, as well as antibodies in the sera of group A mothers and reactive for group B cells, generally do not readily pass the placental barrier, as shown by the fact that they are usually not found in significant amount in the cord sera. On the other hand, antibodies in the sera of group O mothers and reactive for group A cells and group B cells do readily pass the placental barrier as shown by their presence in large amounts in cord sera. These observations indicate a difference between these antibodies and support the theory that the cross-reacting antibodies found exclusively in human group O serum and which is generally of the placental passing variety, is distinct from anti-**A** and anti-**B**, thus justifying the use of the designation anti-**C**. Furthermore, it is evidence against the idea that the cross reactions obtained with group O serum are due to a linkage of anti-**A** and anti-**B** because the linkage hypothesis would imply that two antibodies linked together can more readily traverse the placenta than each can individually.

In heterospecific pregnancies, for example, mother group O and baby either group A or group B, theoretically anti-**C** which has traversed the placenta should be absorbed by the baby's cells and therefore be absent from the baby's serum. In cases of this category, free anti-**C** was, however, demonstrated not infrequently in cord serum. This indicates that anti-**C** is an antibody of low avidity. The relatively low avidity of **C** antibodies probably accounts for the mildness of most cases of erythroblastosis in instances of A-B-O incompatibility, and the total freedom of the disease in still other cases.

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THE RESPONSE OF SERUM INORGANIC PHOSPHORUS AND POTASSIUM TO ORAL GLUCOSE IN NORMAL INDIVIDUALS AND IN PATIENTS WITH MILD DIABETES

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INTEREST has been recently renewed in the alterations of serum phosphorus and potassium following glucose loading intravenously.[1, 2] The decrease in the concentration of serum inorganic phosphorus, which occurs to a greater extent in normal individuals than in diabetic patients, has been suggested as a means of diagnosing diabetes mellitus. A decrease in phosphorus concentration may occur following the oral administration of glucose, but McCullagh[3, 4] was unable to confirm an earlier report of its diagnostic value in distinguishing varying degrees of carbohydrate abnormality. In the normal dog, the concentration of serum potassium decreases and parallels phosphorus in its behavior, following the intravenous administration of glucose; in the pancreatectomized dog, alterations in the concentrations of potassium were found to be variable.[5] Wilson and associates[2] found a greater decrease in the concentration of potassium in diabetic than in nondiabetic persons after the intravenous administration of glucose.

A study was undertaken to learn whether changes in the concentration of serum potassium and phosphorus following the oral administration of glucose would be of value in the diagnosis of mild diabetes mellitus.

METHOD

Included in the study were: (1) known mildly diabetic patients under dietary control alone whose random blood sugar concentrations were normal or slightly elevated; and (2) normal clinic patients and laboratory personnel. They were instructed to eat liberally (at least 200 grams of carbohydrate) for three days prior to testing. Following an overnight fast, each subject drank 100 grams of glucose dissolved in 250 ml. of water. Fasting and 1-, 2-, and 3-hour specimens of blood were taken.

Whole blood was used for the glucose determinations; the serum was separated for the analyses of potassium and phosphorus. The glucose curves were consistent with the initial diagnoses.

The determinations of the concentrations of serum potassium were made on a Baird flame photometer using the internal standard procedure. Phosphorus was determined by a slightly modified Fiske-Subbarow method.[6] Glucose was measured by the Nelson procedure.[7] Values reported are the average of duplicate determinations.

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Received for publication, May 26, 1954.

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RESULTS

Following the oral administration of glucose, concentration of serum potassium decreased in both the mildly diabetic and normal subjects (Table I). The change between the fasting specimen and the lowest value observed after glucose was 0.34 (± s.e. 0.04) meq. per liter in the diabetic subjects and 0.35 (± s.e. 0.08) meq. per liter in the normal subjects; the percentage change in the former was 7.3 and in the latter 9.2. The mean initial concentration of serum potassium was slightly greater in the diabetic (4.62 meq. per liter) than in the normal subjects (4.39 meq. per liter). This difference was observed throughout the glucose tolerance test. No change or increase in the concentration of potassium occurred in one normal and six diabetic subjects.

TABLE I. POTASSIUM CONCENTRATION IN THIRTY-SEVEN DIABETIC AND SIXTEEN NORMAL SUBJECTS IN THREE-HOUR ORAL GLUCOSE TOLERANCE TEST: CHANGE BETWEEN FASTING SPECIMEN AND LOWEST VALUE OBSERVED AFTER GLUCOSE

CHANGE (MEQ./1)	DIABETIC SUBJECTS		NORMAL SUBJECTS	
	NO.	PER CENT	NO.	PER CENT
No change	3	8.1	1	5.6
Elevation	3	8.1	–	–
Decrease				
0.01-0.19	4	10.8	3	16.7
0.20-0.39	12	32.5	6	33.3
0.40-0.59	8	21.6	4	22.2
0.60-0.79	3	8.1	4	22.2
0.80 and above				
Total	37	100.0	18	100.0
Mean	0.34		0.35	
Stand. Dev.	0.26		0.21	
Stan. Error Mean	0.04		0.08	
Mean Per Cent Change	7.30		9.2	

The concentration of phosphorus decreased to the same degree in both groups (Table II). The change between the fasting specimen and the lowest value observed after glucose was 0.51 (± s.e. 0.06) mg. per 100 ml. in the diabetic subjects and 0.52 (± s.e. 0.07) mg. per 100 ml. in the normal subjects, a percentage change of 15.4 and 14.2, respectively.

TABLE II. PHOSPHORUS CONCENTRATION IN THIRTY-SIX DIABETIC AND TWENTY NORMAL SUBJECTS IN THREE-HOUR ORAL GLUCOSE TOLERANCE TEST: CHANGE BETWEEN FASTING SPECIMEN AND LOWEST VALUE OBSERVED AFTER GLUCOSE

CHANGE (MG./100 ML.)	DIABETIC SUBJECTS		NORMAL SUBJECTS	
	NO.	PER CENT	NO.	PER CENT
No change	–		–	
Elevation	1	2.8	–	
Decrease				
0.01-0.39	13	36.1	9	45.0
0.40-0.79	16	44.4	7	35.0
0.80-1.20	5	13.9	2	10.0
1.20 and above	1	2.8	2	10.0
Total	36	100.0	20	100.0
Mean	0.51		0.52	
Stan. Dev.	0.36		0.33	
Stan. Error Mean	0.06		0.07	
Mean Per Cent Change	15.40		14.20	

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DISCUSSION

Glucose administered orally did not produce the same changes in the serum concentration of potassium and inorganic phosphorus as had been reported to follow the intravenous administration of glucose. In the latter instance, the concentration of serum potassium decreased more and that of phosphorus decreased less in the diabetic than in the nondiabetic subjects.[1, 2] However, Gundersen, Bradley, and Marble[8] have just reported a study of the concentrations of serum phosphorus and potassium after the intravenous administration of glucose to mild diabetic patients and normal individuals in which there was no significant decrease in the concentration of either electrolyte in the two groups. The decreases in serum potassium averaged 0.33 meq. per liter in the patients with normal glucose tolerance curves and 0.32 meq. per liter in those with diabetic curves. These decreases are almost identical with those observed after the oral administration of glucose. Also, the decreases in the phosphorus levels after the oral administration of glucose were similar to those observed after the intravenous administration of glucose in both groups.

SUMMARY

Glucose (100 Gm.) was administered orally to thirty-seven mildly diabetic and twenty normal subjects. In both groups the concentration of potassium and phosphorus in the serum decreased to the same degree. The changes in the concentration of these electrolytes following the oral administration of glucose are not of differential value in the diagnosis of mild diabetes mellitus.

Statistical assistance was rendered by Barbara Glenn.

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MILD FAMILIAL HYPOPROCONVERTINEMIA

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INTRODUCTION

WITHIN the last several years our knowledge of the mechanism of blood clotting has been greatly increased by the identification of several new factors and the elucidation of their role in blood coagulation. Cases of bleeding due to lack of various clotting elements have been reported. If some of the cases originally described as "idiopathic hypoprothrombinemia" could be reinvestigated they would in reality be ascribed to a lack of one of the new factors.[1] Only a few case reports of lack of proconvertin have appeared in the literature.[1-6] In some of these, a familial finding of a minor degree of deficiency was noted.

The present study concerns a family in whom a lack of proconvertin (Factor VII, co-thromboplastin, serum prothrombin conversion accelerator [SPCA], stable factor [SF])[7] exists in the latent form.

H. H., a 38-year-old white woman, was admitted to the Albert Einstein Medical Center, Southern Division, on Nov. 17, 1953, with signs and symptoms of acute calculus cholecystitis. The prothrombin time on admission was 15.8 seconds (54 per cent). Between November 20 and November 27, she was given without avail a total of 576 mg. of vitamin K, 200 mg. of vitamin K_1 oxide, and two blood transfusions in order to raise the prothrombin time. The prothrombin time continued to range between 15.8 and 17.6 seconds (54-42 per cent). On November 27, a cholecystectomy was performed. The gall bladder revealed chronic exacerbating gangrenous cholecystitis and cholelithiasis. The other coagulation studies carried out preoperatively were essentially negative (Table I).

TABLE I. ROUTINE COAGULATION TEST

	H. H. (PATIENT PRESENTED)	NORMAL
Clotting Time (Lee White) Three-Tube Method	8-11-14 minutes	12-20 minutes (in third tube)
Prothrombin Time	Average 17.1 seconds	12-13 seconds (100-80 per cent)
Recalcification Time	185 seconds	100-150 seconds
Platelets	240,000 per cu. mm.	200-400,000 per cu. mm.
Clot Retraction	Good	

No unusual bleeding was encountered at the operation. Postoperatively, the patient developed atelectasis of the right lower lobe. This gradually cleared and she was discharged in a healthy state Dec. 17, 1953.

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Received for publication, June 4, 1954.

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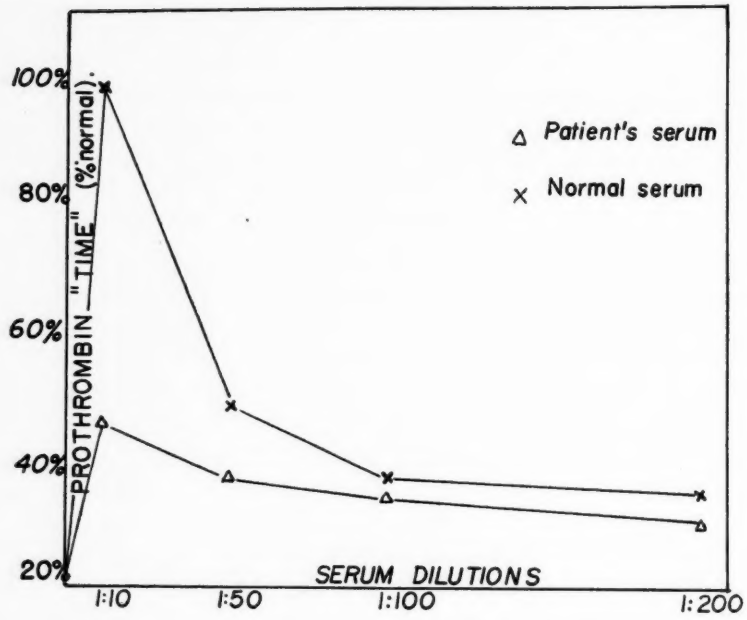

Fig. 1.—Effect of patient's serum on Dicumarolized plasma with prothrombin time of 18 per cent (28 seconds).

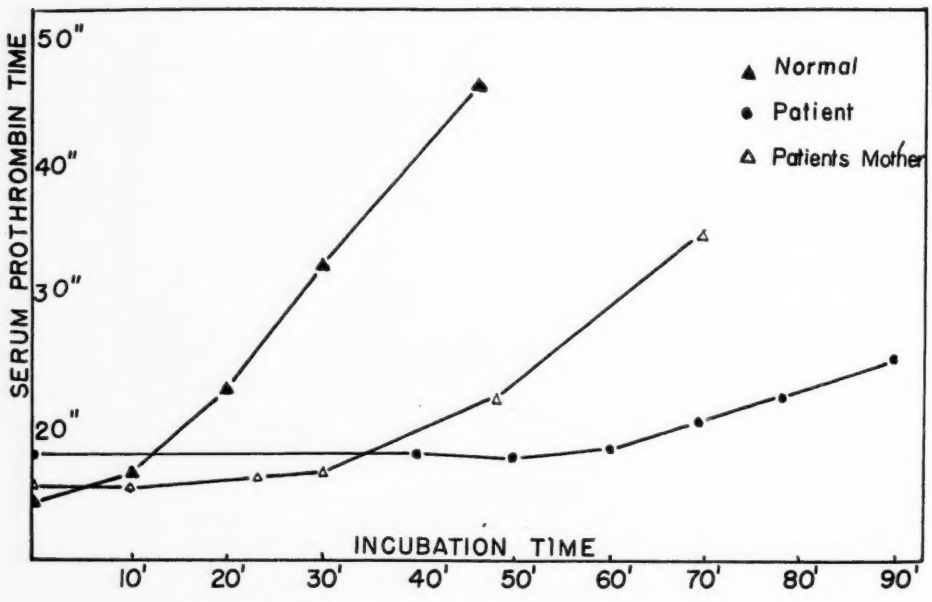

Fig. 2.—Prothrombin consumption test on patient and mother.

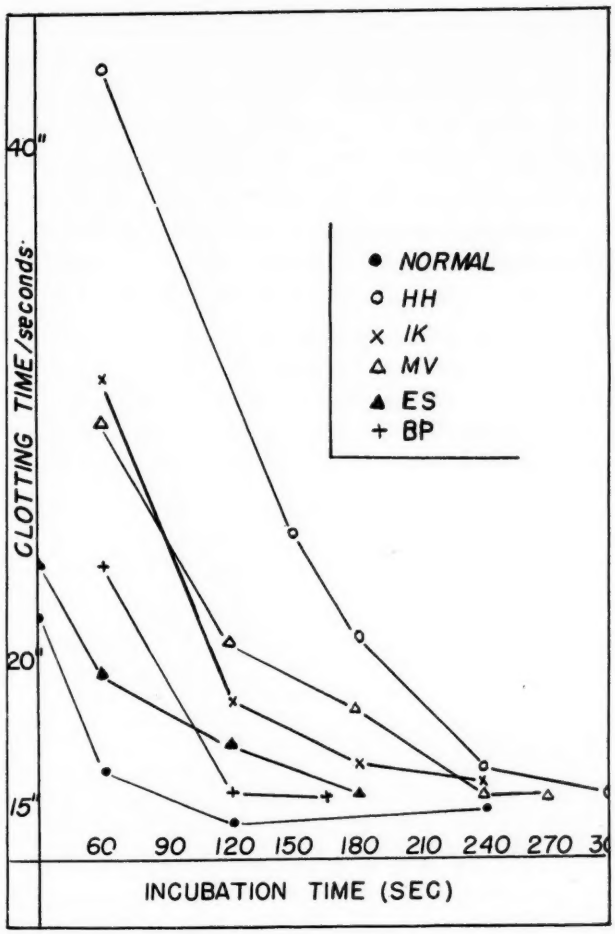

Fig. 3.—Modified two-stage prothrombin time on members of the family.

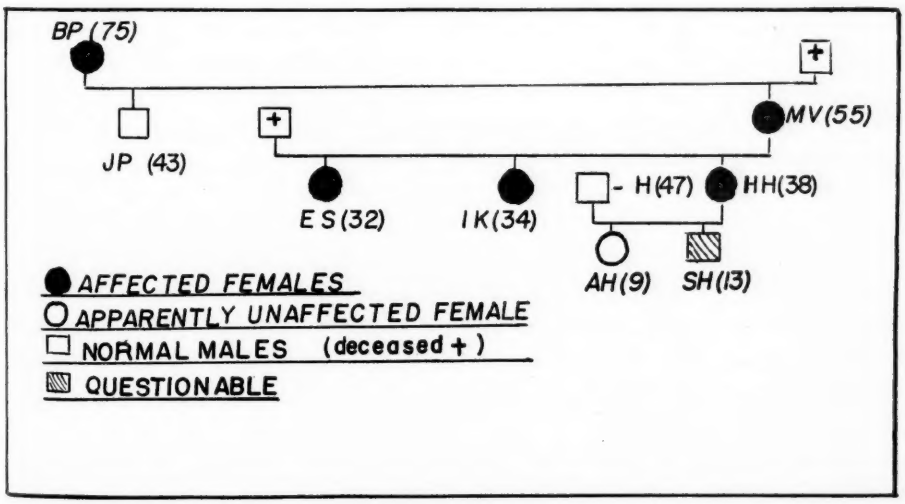

Fig. 4.—Genealogy table.

Past medical history failed to reveal any episodes of bleeding except for one occasion of epistaxis at 13 years of age when she had active rheumatic fever. At her work—candy wrapper—she frequently cuts a finger, but bleeding stops in a short time. The menstrual history is normal.

The family history reveals her mother had a cholecystectomy; one sister (I. K.) had two cesarean sections for disproportionment and a radical mastoidectomy. In none of these instances was bleeding a factor. There is no other family history of bleeding tendencies.

Laboratory Studies.—In the study to determine the factor or factors lacking in this patient's coagulation mechanism it was found that the addition of aged native serum[8] would correct the one-stage prothrombin time[9] (Table II). The addition of the patient's plasma to ancient normal oxalated plasma would correct the prothrombin time of the latter.[10] Since ancient plasma is deficient in Factor V, its correction is evident that the patient had sufficient Factor V.

TABLE II. EFFECT OF ADDITION OF AGED NORMAL SERUM ON PROTHROMBIN TIME

PATIENT*	B. P.	J. P.	M. V.	I. K.	E. S.	H. H.	H.	A. H.	S. H.	NORMAL CONTROL
Age	75	43	55	34	32	38	47	9	13	–
Prothrombin Time	15.7″	12.9″	15.8″	17.8″	15.8″	18.3″	13.0″	13.0″	14.0″	13.0″
Correction by Aged Serum	13.9″	–	13.1″	13.8″	14.2″	12.8″	–	–	13.4″	–

*See Fig. 4 for relationship of the various subjects.

Plasma with Dicumarol is deficient in proconvertin.[11] The addition of aged native serum will restore the prothrombin time to normal.[8] The addition of the patient's serum to the plasma with Dicumarol did not correct the latter (Fig. 1).

The prothrombin consumption test[12] revealed poor utilization of prothrombin (Fig. 2). Hypoproconvertinemia induced by Dicumarol therapy also gives poor utilization of prothrombin.[13]

A modified two-stage prothrombin test[13] revealed adequate amounts of prothrombin but that the conversion to thrombin was delayed (Fig. 3).

A similar defect of variable intensity was observed when the plasmas of the patient's maternal grandmother, mother, and two sisters were tested. The one male on the maternal side had a normal coagulation mechanism.

DISCUSSION

Proconvertin after being converted to plasma convertin is needed to form thrombin from prothrombin. In its deficiency thrombin is not formed, or the formation is so delayed that prolongation of the prothrombin time results. In the family presented, the level of proconvertin is such that while clinical signs are absent it is low enough to give an abnormally long clotting time in the one-stage prothrombin test.

The genealogy of the family is not complete enough to make any statements as to whether we are dealing with a dominant, a recessive, or a sex-

linked phenomenon, though Frick and Hagen[1] believe that hypoproconvertinemia is probably caused by a dominant gene. It is of interest that of three affected generations only females are involved (Fig. 4). But in these three generations only one male is present (and he has had a cerebral thrombosis). In the fourth generation the one female (aged 9 years) tested was not affected. The one male in this generation has a questionably prolonged one-stage prothrombin time.

The lack of correction by vitamin K is consistent with other reported examples of congenital lack of this factor.

SUMMARY

1. A family is presented in whom all the females for three generations have a lack of proconvertin.

2. The literature on hypoproconvertinemia is reviewed.

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THE BLOOD-CLOTTING MECHANISM DURING NORMAL PARTURITION

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HEMORRHAGE during childbirth is usually attributed to uterine atony, premature separation of the placenta, vaginal or cervical lacerations, or, more rarely, to incomplete delivery of the placenta.[1] Bleeding resulting from these causes, however, is not ordinarily lethal. During the last few years it has become evident that fatal hemorrhage is usually associated with profound abnormalities in the blood-clotting mechanisms. It would be of interest to learn whether these changes are an exaggeration of alterations which take place normally. With this in mind, a study was made of certain facets of the clotting process during uncomplicated childbirth in normal mothers. The only consistent change detected was the regular appearance, several hours post partum, of increased spontaneous fibrinolytic activity in vitro, although in some patients considerable variations occurred in other modalities as well. These results confirm the recently published studies of Margulis, Luzadre, and Hodgkinson[2] concerning rapid fibrinolysis post partum.

METHODS

Blood was drawn at intervals from the antecubital veins of women in labor on the Obstetric Service of the University Hospitals of Cleveland, using No. 18 gauge needles coated with tris (2 hydroxyethyl) dodecyl ammonium chloride* and syringes triple-coated with silicone (Desicote, Beckman). The patients were selected because of the absence of complications. As a matter of expediency, only a few of the tests to be described were performed on any one patient.

Determination of the Clotting Time.—Clotting times were performed by a modification of the method of Lee and White.[3] A minimum of 10 ml. of blood was drawn with as little trauma as possible. The needle was removed from the syringe and 1 ml. of blood was then transferred to each of three dry Pyrex or silicone-coated tubes (internal diameter, 11 mm.). The tubes were closed with rubber stoppers, and observed for clotting in water baths at 25° or 37° C. The glass tubes were left undisturbed for five minutes, after which the first tube was tilted once a minute until its contents had clotted. In turn, the second and third tubes were tilted until clotting occurred. The silicone-coated tubes were left untouched for fifteen minutes, and then tilted every ten minutes. Arbitrarily, the end point was considered to be the time no blood ran down the side of the tube when it was tilted, and the time that the third tube clotted was recorded as the *clotting time.*

Determination of Plasma Fibrinogen.—The concentration of fibrinogen in plasma was determined by a method previously described.[4]

Determination of Plasma Prothrombin.—The prothrombin time was determined at 37° C. by one-stage method, mixing equal parts of oxalated plasma, rabbit brain throm-

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This study was supported in part by a research grant (P. H. S. No. H-1661C) from the National Institutes of Health of the United States Public Health Service, and in part by a grant from the Cleveland Area Heart Society.

Received for publication, June 14, 1954.

*Armour Triple Coating Solution was obtained through the courtesy of The Armour Laboratories, Chicago, Ill.

boplastin (Cappell), and 0.025 M calcium chloride solution, in glass tubes (internal diameter, 8 mm.). The oxalated plasma was prepared by mixing nine parts of venous blood with one part of 0.1 M sodium oxalate solution, and removing the cells by centrifugation at 2,500 r.p.m. for fifteen minutes at room temperature in an International SB Size 1 centrifuge. The prothrombin time was also determined on plasma diluted eight times with prothrombin-deficient plasma, prepared by incubating human oxalated plasma for ten minutes at 37° C. with one-tenth its volume of powdered barium sulfate (Baker), and then centrifuging the mixture at 2,500 r.p.m. for fifteen minutes. A more specific test for prothrombin was performed by Alexander's modification[5] of the method of Owren and Aas[6] in which the concentration of prothrombin is determined in the presence of accelerator globulin and serum prothrombin conversion accelerator.

Determination of Thrombin Time.—The thrombin time, that is, the time required for the coagulation of a mixture of oxalated plasma and bovine thrombin, was measured at 37° C. in Pyrex tubes (internal diameter 8 mm.).[7] To compare pregnant and nonpregnant individuals, the results were interpolated relative to a nonpregnant standard thrombin time of thirty seconds.[7] This interpolation was not needed to observe changes during the course of parturition.

TABLE I. THE EFFECT OF FULL-TERM PREGNANCY UPON THE CLOTTING TIME, PLASMA FIBRINOGEN AND PROTHROMBIN ACTIVITY, AND PLATELET COUNT

TEST	NUMBER OF OBSERVATIONS	AVERAGE	RANGE	STANDARD DEVIATION OF MEAN
Clotting time, 25° C., glass tubes (min.)				
Nonpregnant ♀	41	31.2	11-52	9.9
Parturient ♀	21	28.6	20-43	7.0
Clotting time, 25° C., silicone tubes (min.)				
Nonpregnant ♀	21	103.3	71-152	19.5
Parturient ♀	17	113.8	85-142	17.1
Clotting time, 37° C., glass tubes min.)				
Nonpregnant ♀	16	21.1	16-29	3.9
Parturient ♀	14	18.4	13-29	4.6
Plasma Fibrinogen Concentration (mg. per 100 ml.)				
Nonpregnant ♀	36	294	164-485	58.8
Parturient ♀	18	451	327-551	71.1
Plasma Prothrombic Activity* (per cent of pooled normal plasma)				
Nonparturient ♀ and ♂	10	102	88-120	10.1
Parturient ♀	15	121	100-158	15.0
Platelet Count (per cu. mm. whole blood)				
Nonpregnant ♀	12	246,300	175,500-320,000	47,000
Parturient ♀	15	269,300	199,500-344,500	48,500

*Method of Owren and Aas[6] and Alexander.[5]

Determination of Clot Lysis Time.—Three-tenths milliliter of oxalated plasma and 0.3 ml. of bovine thrombin solution (Parke, Davis & Company) containing 10 National Institutes of Health units for milliliter were mixed in Pyrex tubes (internal diameter 11 mm.). After clotting took place, a drop of toluol was added, the tubes were closed with rubber stoppers, and incubated at 37° C. until the clots reliquefied. The length of time until complete dissolution of the clot was recorded as the *clot lysis time.* The minimum recorded time was one day, that is, overnight.

Enumeration of Platelets.—Platelet counts were performed on finger tip blood by the direct method, as modified by Pohle.[8]

Determination of Plasminogen.—The total amount of plasminogen in plasma cannot be estimated accurately because of the presence of inhibitory substances. A relative estimate of the amount of plasminogen in different samples of the same patient's plasma was made by a method previously described in which plasminogen is activated by streptokinase, a substance found in sterile filtrates of cultures of certain hemolytic streptococci.[9]

Determination of Inhibitory Activity of Plasma against Plasmin.—The inhibitory activity of plasma against plasmin, a proteolytic enzyme of plasma activity at or near neutrality, was tested against purified bovine plasmin* by a technique described earlier, in which bovine fibrinogen† was used as a substrate.[10]

RESULTS

1. *Clotting Time.*—The clotting time of venous blood was determined at 25° C. in twenty-one parturient women, and at 37° C. in fourteen others, within a few hours before delivery. Under these conditions, the clotting time did not differ significantly from that of normal nonpregnant women (Table I).

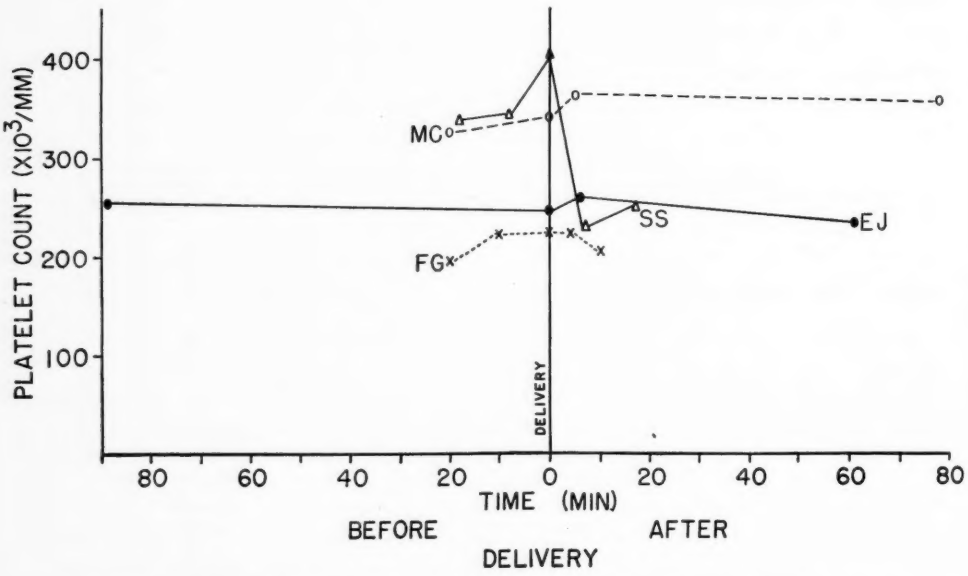

Fig. 1.—Changes in the platelet count during labor in four normal pregnant women. In this and the following figures, time is measured from the moment that the head was delivered. In every case, the first determination thereafter coincided with the delivery of the placenta.

In fifteen subjects, the clotting time was tested at intervals before, during, and after delivery, using both glass and silicone-coated tubes. No signficant variations in the clotting time were noted. In two of these subjects, delivery was by cesarean section. The data obtained in one typical subject are presented in Table II.

2. *Platelet Counts.*—The platelet count, performed before delivery in fifteen women in labor, did not differ from that obtained in nonpregnant women in this laboratory (Table I). Platelet counts were performed serially

*Bovine plasmin (fibrinolysin) was provided through the courtesy of Parke, Davis & Company, Detroit, Mich.

†Bovine fibrinogen was provided through the courtesy of Dr. Walter Seegers, Detroit, Mich.

during labor in eight patients. In seven, there were slight fluctuations in the platelet count during the immediate pre- and postdelivery periods. In the eighth, the platelet count fell precipitously immediately after delivery. The data from this patient and three others are plotted in Fig. 1.

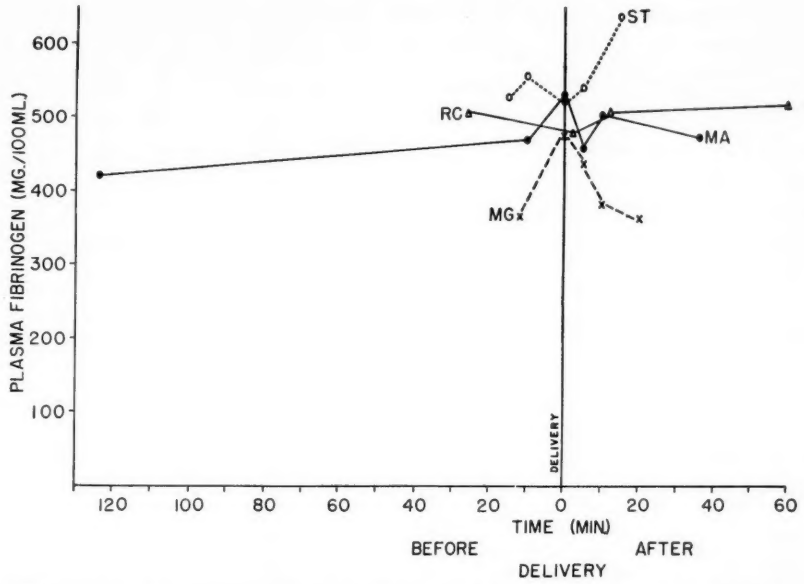

Fig. 2.—Changes in the concentration of fibrinogen during labor in four normal pregnant women.

3. *Plasma Fibrinogen.*—In eighteen parturient women, the concentration of fibrinogen in the plasma averaged 451 mg. per 100 ml. before delivery, in contrast to an average of 294 mg. per 100 ml. in nonpregnant women. (Table I.) During the course of labor, the concentration of fibrinogen fluctuated

TABLE II. THE CLOTTING TIME DURING NORMAL PARTURITION

STAGE OF LABOR	CLOTTING TIME	
	GLASS	SILICONE
50 min. prepartum	25	90
Fetal head delivered	24	78
66 min. post partum	21	84
25 hours post partum	21	80

Clotting time (min.) at 25° C. in glass- and silicone-coated tubes, at intervals during parturition.

greatly, and in no regular relationship to the events of delivery. The changes in the concentration of fibrinogen in four of the twelve patients studied serially are plotted in Fig. 2. In no instance did the fibrinogen fall below 279 mg. per 100 ml. at any time during the course of labor.

4. *Plasma Prothrombic Activity.*—The one-stage prothrombin time was performed in nineteen patients within the last hours before delivery. By this technique, the prothrombin time of undiluted plasma did not differ ap-

preciably from normal in any case. However, when such plasma was diluted eight times with normal barium sulfate-adsorbed plasma, the prothrombin time was often appreciably faster than that of normal plasma. Barium sulfate adsorbs not only prothrombin, but also serum prothrombin conversion accelerator (convertin), the concentration of which affects the prothrombin time. For this reason, the concentration of prothrombin was determined by a more specific method in which excess accelerator globulin and serum prothrombin conversion accelerator were added. Using this method, the concentration of prothrombin appeared to be somewhat higher in the plasma of parturient women than in nonpregnant subjects. With this method, the prothrombic activity in the plasma of fifteen parturient women averaged 21 per cent of normal. (Tables I and III.) When the prothrombic activity of plasma was tested at frequent intervals during the course of labor, no significant changes were observed.

TABLE III. PLASMA PROTHROMBIC ACTIVITY OF PARTURIENT AND NONPREGNANT WOMEN

SUBJECT	ONE-STAGE PROTHROMBIN TIME		"SPECIFIC" PROTHROMBIN TEST
	UNDILUTED PLASMA	PLASMA DILUTED TO 12.5 PER CENT*	
pool of 3 normals	*sec.*	*sec.*	*per cent* 100
Au Normal ♀	16.2	31.8	88
W Normal ♀	14.0	28.4	120
Kr Normal ♀	14.8	27.4	105
B Parturient ♀	14.4	26.0	120
Ar Parturient ♀	14.8	25.0	120
H Parturient ♀	13.8	22.2	158
Kg Parturient ♀	15.6	22.6	144

*With barium sulfate-adsorbed plasma from a nonpregnant subject.

5. *Plasma Thrombin Time.*—The clotting time of a mixture of oxalated plasma and bovine thrombin was measured in sixteen normal pregnant women prior to delivery. In normal nonpregnant individuals, the thrombin time rarely varied by more than three seconds from an arbitrary normal of thirty seconds. Similarly, in these parturient subjects the thrombin time ranged from twenty-eight to thirty-three seconds compared with a thrombin time of thirty seconds in simultaneously studied normal nonpregnant individuals. During labor, no significant changes were observed in the thrombin time in seven women studied serially.

6. *Clot Lysis Time.*—The length of time which elapsed until the lysis of a clot of oxalated plasma and bovine thrombin took place was studied in nine patients. The clot lysis time before the delivery of the infant varied from three to seven or more days, with a median time of five days. In fifteen normal adults, the clot lysis time varied from one to seven or more days, with a median time of four days. Thirteen of these fifteen normal plasma clots lysed in three or more days. The clot lysis time was studied serially in all nine patients. In every case, the clot lysis time shortened in the hours after delivery, usually to a minimum of one day, about two to four hours post partum. The data from three of the nine cases are plotted in Fig. 3. The short-

ened clot lysis time could not be correlated with any change in the concentration of plasminogen, the precursor of the fibrinolytic enzyme of plasma, nor in the inhibitory activity of the patient's plasma against active plasmin. Furthermore, the shortened clot lysis time was independent of the concentration of fibrinogen in the patient's plasma, and therefore of the concentration of fibrin substrate in the clotted plasma. Thus, the mechanism which resulted in the shortened clot lysis time after delivery was not clarified.

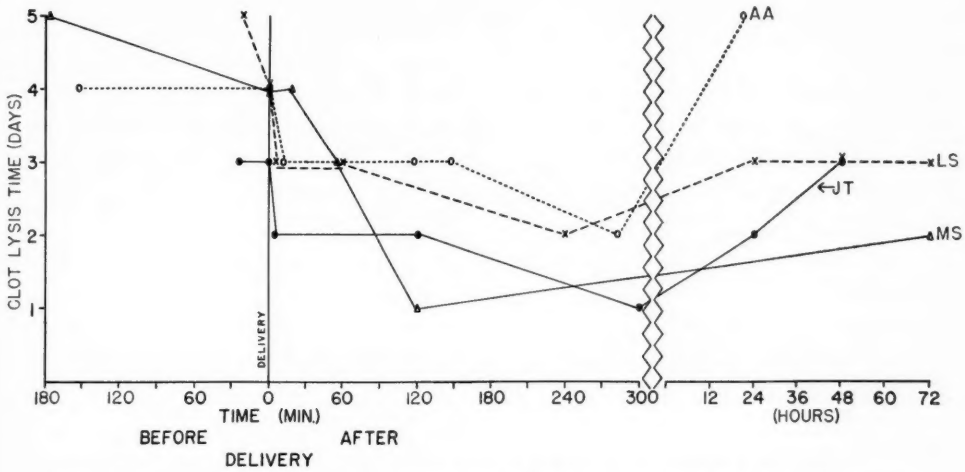

Fig. 3.—Changes in the clot lysis time during labor in four normal pregnant women.

DISCUSSION

There have been many previous studies concerning changes in the clotting mechanism during pregnancy. These have demonstrated that the concentrations of fibrinogen,[11] prothrombin[12-14] and serum prothrombin conversion accelerator[15, 16] are increased in parturient women relative to the nonpregnant state. Similar changes are reported in the present paper, although no data have been provided concerning changes in the concentration of serum prothrombin conversion accelerator.

The concentration of platelets in the circulating blood has been variously reported to be normal,[17] decreased,[18] or elevated[19] in normal pregnant women. With the technique used, we were unable to demonstrate any significant difference between parturient and nonpregnant women. The thrombin time was also normal at the time of delivery, an observation of particular significance, since it is often prolonged in patients with severe pre-eclampsia or eclampsia,[20] Finally, the lysis time of oxalated plasma which had been coagulated with thrombin was normal at the onset of labor.

During the last few years, considerable interest has been expressed in the changes in the clotting mechanism which may occur *pari passu* with uterine hemorrhage associated with labor. Premature separation of the placenta,[21] amniotic fluid embolism,[22] and the intrauterine retention of a dead fetus[23] have all been associated with hypofibrinogenemia as well as with other abnormal-

ities of the clotting mechanism. The present studies suggest that the changes in the clotting mechanisms in these conditions are probably not an exaggeration of changes seen during normal parturition. Slight variations in the concentration of fibrinogen and of platelets occurred during the course of labor. The only consistent change noted in the patients studied was the appearance after delivery of excessive fibrinolytic activity, as measured in vitro, a result which confirms the report of Margulis, Luzadre, and Hodgkinson.[2] The mechanism which results in this rapid lysis was not elucidated; it did not appear to be the result of an increase in the concentration of available plasminogen, a decrease in the inhibtory activity of the plasma against plasmin, nor a change in the amount of available substrate. Other possibilities, such as the appearance of an activator of the enzyme or the deterioration of the inhibitors in vitro were not tested. Similar changes have been observed after severe physical exertion of other types,[24] the injection of adrenalin,[24] or electroconvulsive therapy.[25]

It is noteworthy that the clotting time did not change during parturition even when methods were used which were sensitive to slight alterations in the coagulation mechanisms. Similarly, despite earlier reports to the contrary,[26, 27] it has not been possible to demonstrate significant changes in the clotting time in subjects undergoing strenuous physical exertion.[28] Studies of the clotting time in both of these circumstances are handicapped by the difficulties in obtaining venous blood uncontaminated with tissue juices, and supposed changes must be interpreted guardedly.

SUMMARY

Early in labor, the concentration of fibrinogen and of prothrombin was elevated in parturient women, whereas the clotting time, platelet counts, thrombin time, and clot lysis times were normal. During the course of delivery, the only consistent change observed was the appearance several hours post partum of rapid fibrinolysis, as measured in vitro. No support was obtained for the view that the changes observed in certain hemorrhagic disorders of pregnancy were exaggerations of normal phenomena.

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INCIDENCE OF SEROLOGIC TYPES OF *ESCHERICHIA COLI* ASSOCIATED WITH INFANTILE DIARRHEA AMONG PEDIATRIC PATIENTS IN THE DENVER AREA

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EXAMINATION of the role of certain antigenically defined coli in epidemic and sporadic infantile diarrhea began after 1950 in this country with reports[1, 2] of sporadic cases associated with *Escherichia coli* 0111. Subsequently Modica, Ferguson, and Ducey[3] succeeded in isolating this organism from 45 cases of infantile diarrhea in an outbreak at Grand Rapids, Michigan, in 1951. Of particular interest is the work of Neter and associates[4] in 1952, who reexamined previously frozen stools obtained from two outbreaks in 1947 in upper New York State and recovered *E. coli* 0111 from at least five of the specimens. Because of the paucity of epidemiological data which would link colitypes with infantile diarrhea in this country, there has been considerable preoccupation by American workers with human volunteer feeding experiments. This work, performed on adults, demonstrated that an inoculum of the order of 9,000 million organisms was capable of reproducing gastroenteritis symptoms, cramps, diarrhea, and vomiting.[5, 6] Complicating the picture are reports from abroad[7, 8] suggesting that *E. coli* 0111 and the organisms of Giles,[9] *E. coli* 055, occasionally occur as normal enteric flora. To date there have been no studies made to assess the normal incidence of these organisms in a group of infants and children without diarrhea or suffering the occasional nonepidemic form of the disease. This report deals with such a survey of the pediatric admissions of several hospitals in the Denver community for not only colitypes 0111 and 055, but at least seven other serologic types which have become suspect as etiological agents in infantile diarrhea.

MATERIALS AND METHODS

Cultures were obtained by rectal swabs from pediatric and nursery patients at Colorado General Hospital, Fitzsimons Army Hospital, Denver Children's Hospital, and the Florence Crittenton Home. In the first two institutions, cultures were procured at intervals from all patients on both pediatric and nursery wards without regard to illness, while specimens from Children's Hospital and the Florence Crittenton Home were all on frank cases of diarrhea.

The methods for culturing and antigenic analysis of colitypes were essentially those of the Enteric Unit, Communicable Disease Center, Chamblee, Georgia, and have been outlined in a pamphlet obtainable from this agency. Three colonies of each *coli* culture isolated in Eosin Methylene Blue Agar or on blood agar plates were transferred to Blood Agar base for biochemical study. In addition a wide swath swabbing of the cultures was made and suspended in saline solution for subsequent antigenic study with typing sera. Selection of

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E. coli for typing was based at the outset on IMViC reactions and the nonfermentation of the carbohydrate, galactinol.* In our hands *E. coli* failed to ferment galactinol[10] while *Klebsiella* and *Aerobacter* strains consistently did so. Eventually it became possible to dispense with the time-consuming IMViC series in favor of the galactinol reaction, and this criterion along with sheen production on EMB ultimately determined whether an organism was to be examined antigenically. Thus the last 300 *E. coli* cultures were checked in this manner. Identification of *E. coli* 0 and B antigens was accomplished in this study with unabsorbed sera† containing both 0 and B agglutinins. Although initially all *E. coli* isolations were tested in individual serum it became necessary as additional sera were received to pool this material. The following polyvalent pools therefore were created:

Lot 1 0111,B4, 055,B5, 026,B6
Lot 2 073, 086B7, 0108
Lot 3 0112, 0119, 0124

Positive agglutination in polyvalent sera was investigated further with the individual component sera.

Reading of agglutinations, both slides and tubes, was performed macroscopically. A positive agglutination was always characterized by large, almost immediate floccules which were seen without magnification. The development of small fine floccules was considered to be nonspecific cross agglutination.

RESULTS

A total of 538 cultures performed on material from an identical number of patients was examined for nonlactose-fermenting enteric pathogens and suspect colitypes over a period extending from July, 1952, to October, 1953. A breakdown of the total cases investigated and the diarrheas that were encountered appears in Table I. It may be noted that males and females were about evenly divided but that most of the specimens were obtained from infants under one year of age.

As indicated, most of these cases represented unselected sampling of hospital pediatric and nursery wards with the exception of an original collection of thirty-three cultures from Colorado General Hospital, and fifty-three cultures from Children's Hospital and the Florence Crittenton Home.

One hundred and twenty-eight diarrheas were encountered in the study. There were no deaths due to the diarrheas and in almost all cases none of the patients was critically ill. *Salmonella paratyphi, Shigella flexneri 3,* and *Shigella Sonnei 1* were the only established enteric pathogens isolated from the 128 diarrheas. All were responsible for diarrhea of more than mild character and were obtained from the pediatric wards of Fitzsimons Army Hospital. Paracolobactrum, Proteus, and Enterococcus organisms were not a significant finding in these cases. No established pathogens or suspect colitypes were obtained from the 410 pediatric patients in the absence of diarrhea.

Four colitypes, 0111, 055, 073 (Canioni), and 086, were cultured from four of the diarrheal specimens. All were from male patients and all except the Canioni culture came from children under one year of age. The 0111 culture, typed from one of the original thirty-three coli cultures from Colorado General

*Galactinol, a recent isolate of the Great Western Sugar Company, was kindly supplied for this study by Mr. R. J. Brown, who, together with Mr. R. Serro, was responsible for its recovery from the sugar beet.

†Sera for this study were graciously supplied by Dr. Wm. Ewing, Communicable Disease Center (CDC), Chamblee, Ga., and by Dr. Joan Taylor, Salmonella Reference Laboratory, Colindale, London, England.

TABLE I. INSTITUTIONAL DISTRIBUTION OF INVESTIGATED CASES AND DIARRHEA

INSTITUTION	CASES INVESTIGATED				CASES OF DIARRHEA		
			TOTAL				
	UNDER ONE YR.	OVER ONE YR.	CASES	NON-DIARRHEA	UNDER ONE YR.	OVER ONE YR.	TOTAL
Colo. Gen. Hosp. (33 orig. cult.)	31 F15 M16	2 F1 M1	33	0	31 F15 M16	2 F1 M1	33
Colo. Gen. Hosp. Fitz. A. Hosp. (unselected sampling)	291 F139 M152	161 F84 M77	452	410	36 F15 M21	6 F2 M4	42
Child. Hosp. (Diarrhea Wd.)	38 F14 M24	7 F5 M2	45	0	38 F14 M24	7 F5 M2	45
Florence Crittenton Home	8 F7 M1	0	8	0	8 F7 M1	0	8
Totals	368 F175 M193	170 F90 M80	538	410	113 F51 M62	15 F8 M7	128

F = female; M = male.

Hospital, originated from a 3-week-old infant who was acutely ill on admission, suffering diarrhea, vomiting, dehydration, albuminuria, hematuria, and jaundice. There were no positive urine or blood cultures on this infant. The 055 and 086 isolations were from diarrheas encountered as part of the unselected sampling at Colorado General Hospital. These were from mild diarrheas. The 055 culture was reported to be a nonmotile variant by C.D.C. and possessed no flagellar antigens. Such a strain had been reported by Giles and Sangster[11] but on examination by Kauffmann was later reported as motile and to possess an H6 flagellar component. The Canioni colitype came from an 11-year-old child with mild diarrhea at Children's Hospital, Denver. Follow-up cultures on the 055, 073, and 086 cases were negative following chloramphenicol therapy and the patients became quickly asymptomatic. The sources of nonlactose-and lactose-fermenting pathogens is given in Table II.

TABLE II. INSTITUTIONAL DISTRIBUTION OF CAUSAL AND PRESUMED CAUSAL AGENTS OF DIARRHEA

INSTITUTION	CASES OF DIARRHEA	NONLACTOSE FERMENTERS	LACTOSE FERMENTERS (E. COLI)
Colo. Gen. Hosp. (Orig. 33 cult.)	33		0111,B4
Colo. Gen. Hosp. (Unselected sampling tech.)	26		055,B5 086,B7
Fitzsimons Army Hosp. (unselected sampling tech.)	16	*Shigella flexneri* 3 *Sh. sonnei* 1 *Salmonella paratyphi*	
Children's Hosp.	45		073
Florence Crittenton Home	8		
TOTAL	128	3	4

DISCUSSION

The failure to recover any of the colitypes suspected of pathogenicity from a group of pediatric and nursery admissions in the absence of diarrhea compares favorably with the findings of Giles and colleagues,[12] who isolated 055 only 10 times from 324 healthy infants, none of whom had diarrhea, and of Taylor and Charter,[13] who isolated but four colitypes, 073, E611, and two 086 strains, from 255 healthy control babies. Likewise, the recovery of only four suspect colitypes from 128 sporadic diarrheas would seem to bear out the impression of Taylor[14] that colitype isolations associated with infantile gastroenteritis in any number occur primarily only in outbreaks of epidemic proportions.

It is possible that the low incidence of pathogenic colitypes encountered in this study might be ascribed to two factors. First it was expedient in the survey to use rectal swabs. This method, excellent for Shigella work, perhaps does not reach the most favorable fecal areas as suggested by Stevenson,[15] who contends that *E. coli* 0111 may be a normal inhabitant of the duodenum or jejunum and is washed down by some other diarrheal incitant.

Second, only 100 specimens were checked with three polyvalent sera which comprised nine monovalent types. Perhaps if all nine sera had been on hand at the outset, recovery of more types would have resulted. Initially, *E. coli* strains were tested only in 0111, 055, and 026 serum. Those coli cultures were then discarded as no other typing was contemplated at that time. In July, 1953, however, Dr. Joan Taylor of the Colindale Salmonella Reference Laboratory, London, sent 073, 086, and 0119 sera, and these were immediately added to the serum pools. *E. coli* 073 and 086 figured prominently in investigations of Charter and Taylor,[16] who found that the former was not uncommon in infants with upper respiratory tract infections. The one isolation of Canioni (073) from the Children's Hospital in Denver, however, was from a male child of eleven who had no respiratory symptoms. *E. coli* 086 (Taylor E990), is apparently the only truly nonmotile coli strain among the suspect pathogenic colitypes. It possesses no flagellar antigens and repeated semisolid passage has not produced motility. The 0119 strain was isolated first by Dr. John Smith[17] in Aberdeen, Scotland, and apparently was the cause of some infantile diarrhea and vomiting in that area. No cases involving this organism have been reported in greater London, but Taylor[14] has reported one outbreak in Liverpool due to 0119.

Three other sera, made from *E. coli* 0108, 0112, and 0124, were obtained from Dr. William Ewing of CDC in August, 1953. Except for 0112, which has been shown to be identical with De Assis' *Shigella guanabara* by Ewing and Kauffman,[18] these strains have not occurred in any infantile diarrhea outbreaks in this country or abroad.

Antiserum for *E. coli* 0124, identical with *Shigella dysenteriae 3* (Q771), was added along with serum for *E. coli* 0108, a strain responsible for many adult diarrheas in Georgia,[19] simply because these represented new colitypes whose pathogenicity for adults was known but whose pathogenicity for a younger age

group had never been assessed. There were no recoveries using these last three sera, but, as previously stated, only 100 cultures were subjected to antigenic analysis with the full complement of nine sera.

Of interest is the fact that all recognized recovered colitypes were from diarrhea cases. In the absence of nonlactose fermenters and excluding virus etiology, it would seem that perhaps these organisms might be regarded as causal agents of the diarrhea. More important however, are the findings of no suspect coli pathogens in the normal stools of the pediatric population. This would lend support to Neter's[2] view that 0111 and 055 do not constitute normal *E. coli* flora and this might now be extended to include 073 and 086. The fact that all diarrheas from which colitypes were isolated occurred in infants under one year of age, with one exception, must be disregarded as statistically insignificant, as should also the fact that these recoveries were all in male children. The recovery rate is much too small and no evidence exists in the literature that male infants are more susceptible than females.

SUMMARY

1. A survey of 538 Denver hospital pediatric and nursery patients ranging in age from less than 7 days to 13 years using the rectal swab technique resulted in the isolation of four antigenically defined colitypes of possible pathogenicity over a period of fifteen months.

2. *E. coli* 0111, 055, 073, and 086 were recovered from the diarrheal cases only of this group and with one exception from infants under one year of age. In the absence of nonlactose fermenters it is possible that these organisms were the cause of these diarrheas.

3. There were no pathogenic colitype isolations from 410 nondiarrhea pediatric cases.

The cooperation of Dr. Robert H. Alway, Professor of Pediatrics, University of Colorado Medical School, and Lt. Col. Elwyn N. Akers, MC, Fitzsimons Army Hospital, is gratefully acknowledged.

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USE OF CYCLOHEXIMIDE* IN THE SELECTIVE ISOLATION OF FUNGI PATHOGENIC TO MAN

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THE cultural isolation of pathogenic fungi from clinical materials is often hindered or prevented by the overgrowth of the medium by contaminating bacteria and saprophytic fungi. This is particularly true of such clinical materials as sputum, exudates from open lesions, skin scrapings, or nail clippings, all of which are apt to be heavily contaminated by saprophytic microorganisms.

EXPERIMENTAL STUDIES

Various procedures have been devised in efforts to control bacterial contamination: lowering the pH of the isolation medium to levels unfavorable to the growth of bacteria, as in the commercially produced Sabouraud dextrose agar (pH 5.6); the addition of antibacterial antibiotics, such as penicillin and streptomycin, to the medium; or the addition of antibacterial dyes.[1]

The growth of saprophytic fungi has been controlled with varying degrees of success by the use of such substances as oxgall,[1] which tends to restrict the spreading, and thus the intermingling, of colonies of both saprophytic and pathogenic fungi, or by raising the pH of the medium to a level that restricts the growth of saprophytic molds to a greater extent than that of the pathogens.[2]

With the discovery by Whiffen and associates[3] of cycloheximide, an antibiotic produced by *Streptomyces griseus*, a new approach toward the control of mold contaminants was made possible. This antibiotic, though completely lacking in antibacterial activity, has the unique property of inhibiting the growth of a wide variety of saprophytic molds; but, with the striking exception of *Cryptococcus neoformans*, it has little or no adverse effect on the development of fungi capable of causing animal infections.

A number of investigators have utilized these properties of cycloheximide to advantage. Phillips and Hanel[4] added cycloheximide to solid media to obtain mold-free cultures of bacteria.

A medium incorporating cycloheximide as well as penicillin and streptomycin was developed by the Mycology Unit of the Communicable Disease Center in its search for a means of selectively isolating *Coccidioides immitis* from both clinical and other materials.[5] Under laboratory conditions, it was possible to separate *C. immitis* from a mixture of spores of fifteen different saprophytic fungi on this medium.

Fuentes and colleagues[6] found cycloheximide useful in the isolation of pathogenic fungi from a variety of clinical specimens, and suggested that it may be of value in purifying stock cultures of human pathogenic fungi contaminated by saprophytic molds.

The value of cycloheximide in isolating dermatophytes from clinical materials was demonstrated by Georg.[7] In a series of cultures made from toenail clippings from persons with suspected onychomycosis, and in a series of cultures of hairs and skin scrapings from cases of suspected ringworm in cattle, three times as many dermatophyte isolates were obtained on a cycloheximide medium as on a similar medium without cycloheximide. In many instances

Received for publication May 24, 1954.

*Distributed under the trade name of "Acti-Dione" by the Upjohn Company, Kalamazoo, Mich. Trade names are used for identification purposes only and their use does not constitute endorsement of the product by the Public Health Service. Acti-Dione used in these studies was kindly furnished by the Upjohn Company.

**From the Communicable Disease Center, Public Health Service, United States Department of Health, Education and Welfare, Atlanta, Ga.

the dermatophytes were obtained in pure cultures. Such clinical materials usually yield few cultures of dermatophytes, due to heavy contamination with saprophytes.

This same medium made possible the recovery by Ajello and Getz[8] of *Trichophyton mentagrophytes, T. rubrum,* and *Epidermophyton floccosum* from used shoes, and of *T. mentagrophytes* from tile in a shower stall.

In the hopes of extending the use of cycloheximide to improve methods for isolation of other pathogenic fungi from clinical materials, the following studies have been undertaken.

EXPERIMENTAL STUDIES

In preliminary studies, several strains of each of the common fungi which cause subcutaneous or systemic mycoses* (with the exception of *Actinomyces bovis,* which has special growth requirements) were grown in test tubes containing 5 ml. Sabouraud dextrose agar† adjusted to pH 6.5 to 6.8, and on the same medium to which cycloheximide in 0.1 mg. per milliliter and 0.5 mg. per milliliter quantities, respectively, had been added. Gross comparison of growth obtained on the three media indicated that the growth of these fungi (with the exception of *C. neoformans*) did not appear to be inhibited by cycloheximide in these concentrations. The test strains of *A. boydii* and *Asp. fumigatus* did, however, show some retardation of growth in tubes containing 2.5 mg. cycloheximide per 5 ml.

In contrast to these findings with the pathogenic fungi, all of twelve species of saprophytic fungi similarly tested in a previous study[7] had shown significant degrees of inhibition by corresponding amounts of cycloheximide.

Quantitative tests were made in order to determine more accurately the sensitivities of both mycelial and yeast phase cultures of these various pathogenic fungi to cycloheximide. Serial dilutions of cycloheximide (sterilized by filtration) were made in tubes containing Sabouraud dextrose broth at pH 6.5. Final volume in each tube was 5 ml. Yeast phase cells were possible; conidia from the mycelial phase, or small pieces of mycelium (*N. asteroides*) of the various test fungi were prepared in dilute suspensions and inocula containing a small number of units (approximately twenty-five cells or mycelial fragments by hemocytometer count) were added to each tube. In the case of *H. capsulatum* it was found necessary to use much larger inocula, approximately 5,000 spores, in order to obtain growth in the control tubes of Sabouraud dextrose broth. All tubes were incubated at 30° C. and read at weekly intervals for three weeks. The amount of growth was estimated as 1+ to 4+ as compared to the growth in two control tubes which were considered 4+ at the time of each reading.

RESULTS

The results of these tests are presented in Table I. The test fungi are arranged from top to bottom in the table in order of their increasing inhibition by cycloheximide. It will be noted that *P. pedrosoi* and *P. compactum,* rather than being inhibited by the antibiotic, were stimulated at the heavier concentrations. This stimulating effect is indicated by denoting the growth as being greater than the 4+ noted in the controls.

The growth of *C. neoformans* was completely inhibited at concentration of cycloheximide ranging from 20 mg. to 0.16 mg. per 5 ml. broth. Additional tests showed that 4+ growth occurred only when the concentration of cycloheximide was lowered to 0.0025 mg. per 5 ml.

**Allescheria boydii, Aspergillus fumigatus, Blastomyces brasiliensis, B. dermatitidis, Candida albicans, Coccidioides immitis, Cryptococcus neoformans, Geotrichum sp., Histoplasma capsulatum, Nocardia asteroides, Philalophora compactum, P. pedrosoi, P. verrucosa, Sporotrichum schenckii.*

†Difco Co., Detroit, Mich., Neopeptone 1 per cent, dextrose 4 per cent, agar 1.5 per cent

TABLE I. EFFECT* OF VARIOUS CONCENTRATIONS OF CYCLOHEXIMIDE ON THE GROWTH OF PATHOGENIC FUNGI

SPECIES	NO. STRAINS TESTED	PHASE†	QUANTITIES OF CYCLOHEXIMIDE IN 5 ML. SABOURAUD DEXTROSE BROTH																NO CYCLOHEXIMIDE	
			20 MG.		10 MG.		5 MG.		2.5 MG.		1.25 MG.		0.63 MG.		0.32 MG.		0.16 MG.			
DAYS INCUBATION AT 30° C.			14	21	14	21	14	21	14	21	14	21	14	21	14	21	14	21	14	21
P. pedrosoi	2	M	6	6	5	6	5	5	4	5	4	5	4	4	4	4	4	4	4	4
P. compactum	1	M	6	6	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
P. verrucosa	2	M	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
C. albicans	2	Y	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Geotrichum sp.	2	M	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
S. schenckii	2	Y	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
C. immitis	3	M	3	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
N. asteroides	2	M	1	1	2	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4
H. capsulatum	3	Y	0	1	1	2	3	3	4	4	4	4	4	4	4	4	4	4	4	4
H. capsulatum	3	M	0	0	1	1	2	3	3	4	3	4	4	4	4	4	4	4	4	4
B. dermatitidis	3	Y	0	0	1	2	2	3	3	4	4	4	4	4	4	4	4	4	4	4
B. brasiliensis	2	Y	0	0	0	1	1	2	2	3	4	4	4	4	4	4	4	4	4	4
A. boydii	2	M	0	1	0	1	1	1	1	1	1	1	1	2	2	2	3	3	4	4
A. fumigatus	2	M	0	0	0	0	0	0	0	1	1	2	2	2	2	3	3	3	4	4
C. neoformans	3	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4

*Growth in cycloheximide tubes estimated as 1+ to 6+ in contrast to that attained in control tubes which was considered as 4+ at the time of each reading.

†Phase: M = Mycelial type culture; Y = Yeast type culture.

Along with *C. neoformans, A. boydii* and *Asp. fumigatus,* which were next in order of sensitivity, would not be expected to be recovered from clinical materials and other substrata with medium containing cycloheximide in amounts which would be effective in the inhibition of saprophytic molds.

On the other hand, all the other test fungi were either resistant to 5 mg. cycloheximide in 5 ml. broth or attained at least 2+ growth in this concentration by the twenty-first day. If in diagnostic work cycloheximide is used at a concentration of 0.5 mg. per milliliter in tubes containing 5 to 10 ml. medium, it is expected from these results that all of the fungi tested, with the few exceptions listed previously, would be recovered.

The effectiveness of cycloheximide in the isolation of *H. capsulatum* was tested by incorporating this antibiotic in the medium used to culture the liver and spleen of mice injected with suspensions of soil known to have been positive for *H. capsulatum.** The details of the method used in this laboratory to isolate *H. capsulatum* from soil have been presented by Ajello and Runyon.[9]

Aliquots of soil suspension prepared from eleven positive soils were injected intraperitoneally into sets of four mice; eight weeks following injection all the surviving mice were sacrificed. A set of four tubes containing 8 ml. of peptone-glucose agar was inoculated with tissue from each mouse as follows: two tubes with single pieces of liver and two tubes with single pieces of spleen, and another set of four tubes of the same medium to which had been added 0.5 mg. per milliliter of cycloheximide.

As shown in Table II, *H. capsulatum* was recovered with the same frequency on both media, indicating that the concentration of cycloheximide recommended for use in the selective isolation medium was not inhibitory to the development of this fungus. As saprophytic fungi in soil inocula are usually destroyed after six to eight weeks in animal tissues, the pathogenic fungus, *H. capsulatum,* was

TABLE II. COMPARATIVE EFFECTIVENESS OF TWO MEDIA IN THE ISOLATION OF H. CAPSULATUM FROM MOUSE TISSUE (FINDING PRESENTED AS RATIO OF POSITIVE TO POTENTIALLY POSITIVE CULTURES OBTAINED FROM THE SURVIVING MICE)

SOIL SAMPLE	NO. OF SURVIVING MICE	PEPTONE-DEXTROSE AGAR*	PEPTONE-DEXTROSE AGAR + CYCLOHEXIMIDE†
		NO. TUBE + FOR H. CAPSULATUM	NO. TUBE + FOR H. CAPSULATUM
22C	4	0/16	0/16
26B	4	4/16	4/16
28B	4	0/16	0/16
35B	2	2/8	4/8
60A	4	16/16	16/16
60C	4	15/16	14/16
61B	4	16/16	16/16
62B	1	0/4	0/4
63B	1	4/4	4/4
63C	3	12/12	12/12
66A	3	0/12	0/12
Totals		69/136 = 50.7%	70/136 = 51.5%

*Dextrose, 1.0 Gm., Neopeptone, 1.0 Gm., distilled water – 100.0 ml., Agar 2.0 Gm. Adjusted to pH 7.0.

†Cycloheximide, 0.5 mg. per milliliter.

*These soils were being retested to determine the longevity of *H. capsulatum* spores in soil.

isolated in pure culture in most instances. The cycloheximide medium, therefore, did not offer the advantage it would afford had the tissues been heavily contaminated with saprophytic fungi.

DISCUSSION

On the basis of previous studies with the dermatophytes and the present sensitivity tests with the pathogenic fungi which produce subcutaneous or systemic fungus disease of man, the use of the cycloheximide medium can be recommended for the isolation of most of the fungi pathogenic to man.

One must bear in mind, however, the few exceptions: *C. neoformans, Asp. fumigatus,* and *A. boydii,* which are sensitive to cycloheximide, as well as *N. asteroides,* which may be sensitive to either penicillin or streptomycin. Because of these exceptions and the possibility of some unusual strain of pathogenic fungus which may be sensitive to one of these antibiotics, it is important always to include a medium without antibiotics in routine isolation from clinical materials. The great advantage of including the antibiotic medium, however, becomes apparent in the increased number of isolations of the majority of the pathogenic fungi which might otherwise have been completely overgrown by saprophytic microorganisms on a less selective medium. The fact that the antibiotics do not alter the gross or microscopic appearance of the fungus cultures, in most instances, allows their ready identification directly on the isolation medium.

The fungi which cause subcutaneous or systemic fungus diseases are not recovered frequently enough in most laboratories to allow comparative studies on the efficiency of any one medium for their isolation from routine clinical materials. We have, however, received a number of reports of the successful use of media containing cycloheximide in the isolation from clinical materials of the following pathogenic fungi: *C. immitis, B. dermatitidis, H. capsulatum,* and *S. schenckii.* In most instances parallel cultures made on the usual media were completely overgrown with saprophytic fungi.

The purification of stock culture of pathogenic fungi by serial transfers on a cycloheximide medium as suggested by Fuentes and associates[6] has also made possible the recovery of numerous stock cultures in the Communicable Disease Center which had been overgrown by saprophytic fungi.

STABILITY OF CYCLOHEXIMIDE

Studies were undertaken to determine the stability of cycloheximide at high temperatures and in storage.

1. Thermostability was tested by preparing a fresh stock solution of 0.5 per cent cycloheximide that was steriliged by Seitz filtration. One portion of the stock solution was kept as a control and two other portions were autoclaved at 110° C. for ten minutes and at 110° C. for twenty minutes, respectively. Tests of the antibiotic activity of the control and autoclaved solutions were made using a strain of *C. neoformans* and serial dilutions of cycloheximide in Sabour-

aud dextrose broth as in previously described sensitivity tests. No difference in activity against *C. neoformans* could be detected between the unautoclaved and autoclaved cycloheximide.

2. Stock solutions stored at 5° C. and 30° C. were tested at biweekly intervals over a six-month period. No difference in antibiotic activity towards *C. neoformans* could be detected between the stock solution held at 5° C. and that kept at 30° C. and no loss of potency occurred in either solution over the six-month period of observation.

FORMULA FOR THE SELECTIVE ISOLATION MEDIUM FOR PATHOGENIC FUNGI

Cycloheximide—Penicillin—Streptomycin Medium

Although cycloheximide may be incorporated into most media, it is recommended that it be used in conjunction with Sabouraud dextrose agar. This medium is preferred as a base not only because it is readily available, but principally because most of the pathogenic fungi are identified on the basis of the gross and microscopic characteristics developed on this medium. Neither cycloheximide, penicillin, or streptomycin alter in any manner the morphology of those pathogens whose growth is not inhibited by their action. Thus, it is not generally necessary to subculture isolates on other media to identify them.

The recommended medium is prepared by autoclaving and cooling Sabouraud dextrose agar to 45° C. and aseptically adding the antibiotics in the following concentrations:

Cycloheximide (Acti-Dione)	0.5 mg. per milliliter
Penicillin	20 units per milliliter
Streptomycin	40 units per milliliter

The complete medium is distributed aseptically into sterile tubes (18 by 150 mm.) in 8.0 ml. amounts and slanted.

To facilitate isolation of the vitamin-deficient dermatophyte, *T. verrucosum*, it is advantageous to add thiamine (0.01 mg. per liter) to this medium. Since both thiamine and cycloheximide are thermostable, these substances may be autoclaved together in the medium before cooling and adding the penicillin and streptomycin.

To simplify the preparation of the medium, a search was made for an antibacterial antibiotic which would survive autoclaving and which could be used in place of the penicillin and streptomycin. Neomycin was thought to fit those requirements, but, though thermostable, it proved unsatisfactory in that it inhibited the growth of several of the pathogenic fungi at the concentrations required to suppress the development of some of the bacteria present in sputum, stools, and other clinical materials.

SUMMARY

Controlled experiments demonstrated that concentrations of cycloheximide (Acti-Dione), which inhibited the growth of many saprophytic fungi, did not

suppress the growth of most of the fungi which produce subcutaneous or systemic disease in man. The following exceptions were noted: *C. neoformans, A. fumigatus,* and *A. boydii.*

To date the cycloheximide medium has been successfully used to obtain pure cultures of pathogenic fungi from sputa, animal hairs, toenail clippings, and other materials heavily contaminated with saprophytic molds and bacteria.

The cycloheximide medium has also proved useful in freeing stock cultures of pathogenic fungi from contaminating saprophytic molds.

This medium should be useful in many phases of medical mycology where the inhibition of contaminating microorganisms is important.

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THE EFFECT OF CONTINUOUS AND INTERMITTENT PRIMAQUINE THERAPY ON THE RELAPSE RATE OF CHESSON STRAIN VIVAX MALARIA

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THE earliest evidence for prophylaxis in vivax malaria was reported for pamaquine by James.[6] Since his original observation, several other 8-aminoquinolines have been found to possess a prophylactic action (Jones and associates[7]). In 1950, Edgcomb and associates[3] reported that primaquine, a compound closely related to pamaquine, gives a fourfold increase in curative activity over pamaquine in man. This comparison of curative activity was done by a highly stylized test in which the disease was allowed to produce the first clinical attack before treatment was instituted. Because the tissue forms of vivax malaria appear to undergo a certain maturation process with time, it is not possible to draw conclusions about the action of a drug against tissue forms in the incubation period from evidence derived from tests performed during the primary attack, such as those reported by Edgcomb. The following studies have for their primary aim an evaluation of the prophylactic action of primaquine.

The standard method for testing causal prophylaxis was devised originally by James[6] and has been used by most later investigators. This method requires that the drug be given on the day prior to inoculation to act in some sort of priming fashion. The drug is then given on the day of inoculation and for six successive days. This program is usually represented by the formula 1-1-6. The biologic justification for this program of drug administration was given many years later by Fairley[4] who found that massive blood subinoculations from an infected volunteer failed to transmit an infection when given to a second volunteer until 192+ hours after the sporozoites were introduced into the first man. This period of 192 hours following a mosquito-induced infection is thus set apart from the other stages of the infection and is called the incubation period. Since trophozoites have not been demonstrated by this

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This investigation was carried out under contract number DA-49-007-MD-20 between the Office of the Surgeon General, United States Army, and the Department of Medicine of the University of Chicago.

This work was further aided by the participation of Army Medical Officers assigned to the project by the Surgeon General of the United States Army.

The investigations reported in this paper would not have been possible except for the cooperation of the inmates and the administrative staff of Stateville Penitentiary.

Received for publication May 25, 1954.

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sensitive technique before the first nine days following a known infection, it is convenient to consider these nine days as the true incubation period. With drug therapy terminated on the sixth day, it is unlikely that a rapidly excreted or degraded drug like primaquine will persist into the period identified by the appearance of the first trophozoites and thereby obscure the results obtained against the early tissue forms.

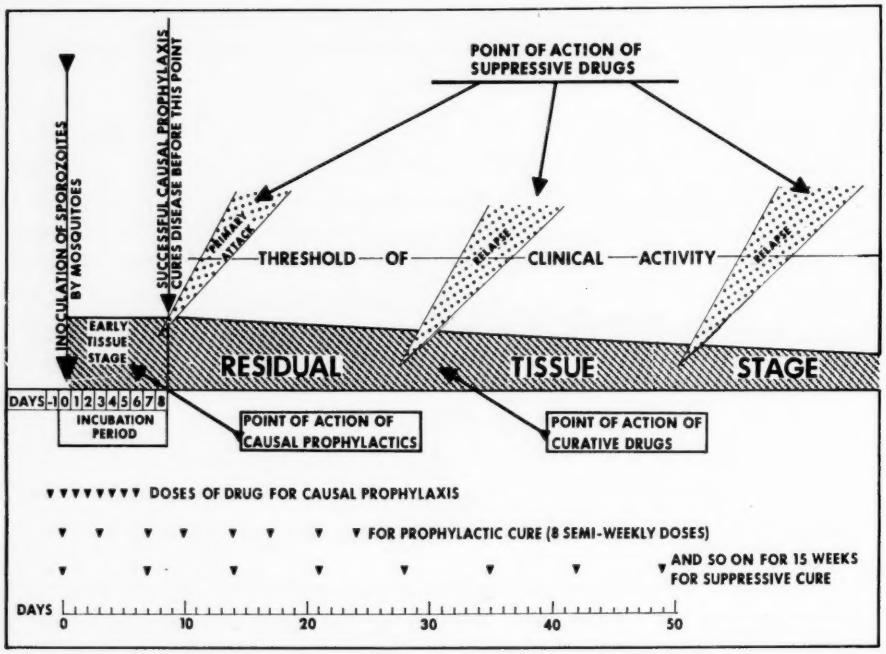


Fig. 1.—Schematic outline of the natural history of Chesson strain *vivax* malaria, demonstrating points of action of "causal prophylactic," "curative," and "suppressive" drugs. The drug administration schedules used in testing for "causal prophylaxis," "prophylactic cure," and "suppressive-cure" are also shown.

In this study a comparison of causal prophylactic activity has been made of quinine, chloroquine, quinacrine, pamaquine, and primaquine using the method of James.[6] A study of prophylactic-cure and suppressive-cure has been made with primaquine alone and in combination with chloroquine. For practical reasons a prophylactic-cure with primaquine alone, and a suppressive-cure with primaquine and chloroquine, have been studied with intermittent doses given either weekly or semiweekly.* These two forms of therapy were begun on the day of inoculation and were continued for from four to fifteen weeks. This method of treatment along with a concept of the natural history of the disease is shown in Fig. 1.

METHODS AND PROCEDURES

Each subject selected for this study was a volunteer from the general inmate population of the Illinois State Penitentiary (Stateville Branch). None of the men had a his-

*The studies of the intermittent administration of primaquine as a means of causing radical termination of vivax infections were stimulated by the unpublished observations of Dr. Leon H. Schmidt (The Christ Hospital, Cincinnati), who had found intermittent therapy effective in cynomologic infections in monkeys.

tory of possible exposure to malaria. The malaria used in these experiments was the Chesson strain of *Plasmodium vivax* and has been used repeatedly in the past because of its ability to relapse early. This is of special advantage in giving an early answer about the effectiveness of a given drug. Each experimental volunteer was bitten in rotation along with at least one control volunteer by ten heavily infected mosquitoes. As a measure of the severity of the infection, we required that the control for each experimental subject must develop parasites detectable on thick smear fourteen days or less after inoculation, since it has been shown that long incubation periods follow light sporozoite inoculations. As a further check on the weight of the infection, each salivary gland was dissected after the mosquito had fed and was graded on a logarithmic scale from 1+ to 4+ depending on the intensity of sporozoite infection. In the past seven years (with over 1,500 attempted infections by this technique) only one out of every 250 untreated volunteers has failed to develop clinical malaria. It is certain that these infections offer a greater therapeutic challenge than most naturally acquired infections.

All drugs were given in single daily doses—a priming dose on the day before inoculation, a second dose on the day of inoculation and a dose on each of the next six days in the *"causal prophylaxis"* experiments—and weekly or semiweekly in the *"intermittent prophylaxis"* and *"intermittent suppressive-cure"* studies. The subsequent follow-up has been described in detail by Alving and associates[1] and includes close clinical supervision and frequent parasite counts. Malaria is not endemic at State Penitentiary, so that accidental infection is not possible.

RESULTS

In order to demonstrate the unique causal prophylactic action of primaquine, a group of three important antimalarial agents were tested for causal prophylactic effect by the procedures outlined previously. These three drugs (quinine, chloroquine, and quinacrine) have been shown to possess remarkably effective action against trophozoites of malaria. That this action in the strain of malaria used is not associated with an effective action against the early tissue stages is shown by the results indicated in Table I. Only one case out of a total of eighty-two treated by one or another of these drugs during the incubation period failed to develop malaria.

TABLE I. THE LACK OF "CAUSAL PROPHYLACTIC" ACTION OF SEVERAL COMMON ANTIMALARIAL DRUGS WHEN USED AGAINST CHESSON STRAIN VIVAX MALARIA

DRUG	PERIOD OF ADMINISTRATION	DOSE (BASE WEIGHT)	PATENT INFECTIONS / NUMBER EXPOSED
Quinine	1-1-6	1.64 Gm./day	4/4
Quinine	1-1-13	1.64 Gm./day	14/14
Chloroquine	1-1-6	0.275 Gm./day	13/13
Atabrine®	1-1-6	0.400 Gm./day	50/51

Because of the similarity of action between pamaquine and primaquine, these two drugs were subjected to a formalized comparison of their causal prophylactic activity. Results of a comparison of primaquine and pamaquine are summarized in Table II. In this series of experiments, both drugs were given in single daily doses (1-1-6). Doses of 31.5 mg. pamaquine base given in this manner failed to protect four out of five volunteers. In divided daily doses it requires 90 mg. pamaquine base to protect three out of five volunteers when given 1-1-6 (Jones and colleagues[7]). Since 90 mg. per day represents the upper limit of tolerance for pamaquine, the dose of pamaquine required

to protect all or nearly all cases from patency is of only academic interest. Primaquine, on the other hand, in single daily doses of 30 mg. base (1-1-6) was able to protect all ten men tested. In doses of 30 mg. primaquine base and 31.5 mg. pamaquine base for eight days, no appreciable toxicity from either drug was observed.

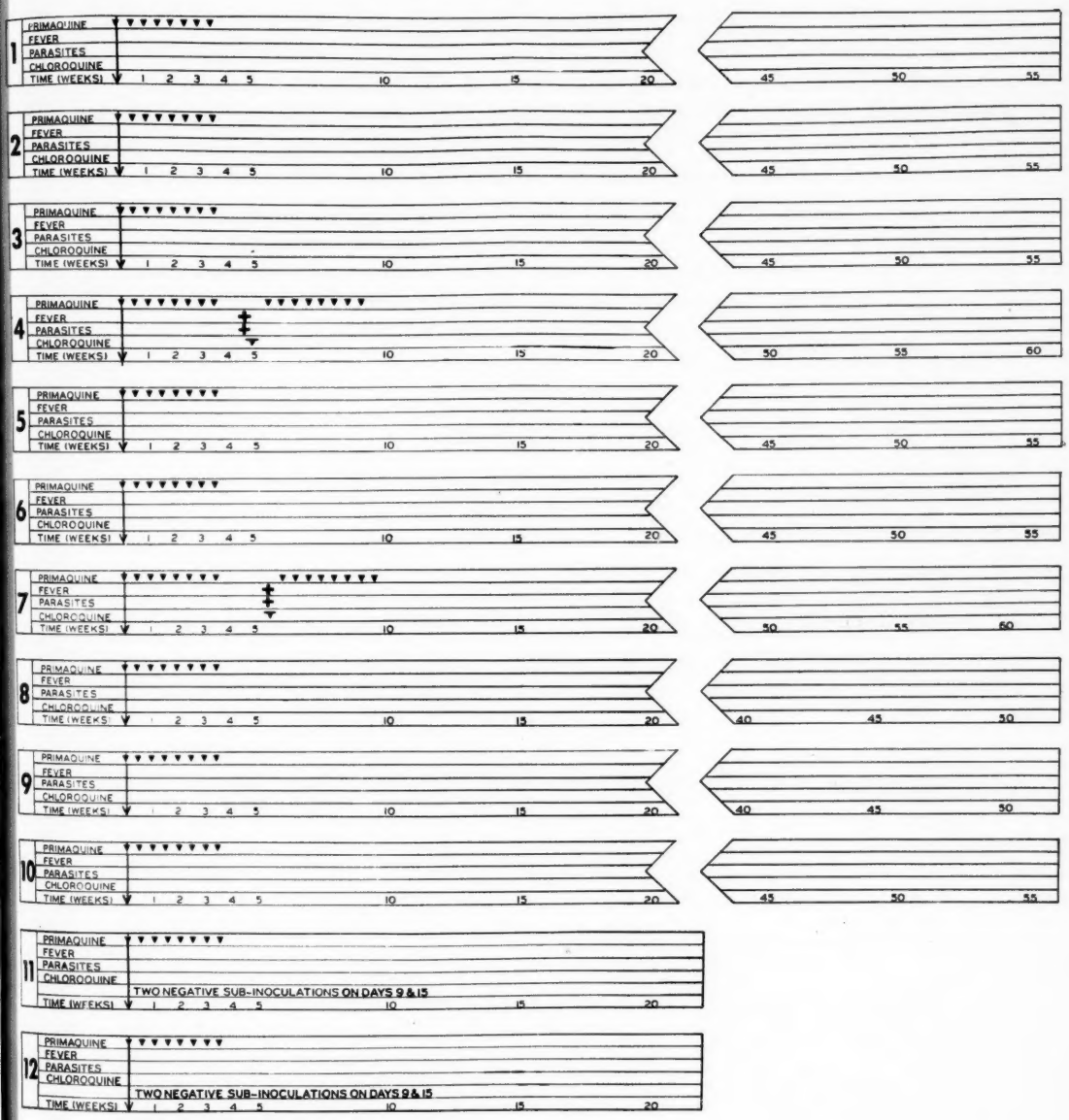
TABLE II. A CONTROLLED COMPARISON OF THE "CAUSAL PROPHYLACTIC" ACTION OF PRIMAQUINE AND PAMAQUINE IN MOSQUITO-INDUCED VIVAX MALARIA (CHESSON STRAIN)

EXPERIMENTAL				UNTREATED CONTROLS
INCUBATION PERIOD (DAYS)		FOLLOW-UP (DAYS)	PATENT INFECTIONS / NUMBER EXPOSED	PATENT INFECTIONS / NUMBER EXPOSED
PARASITES	FEVER			
Pamaquine (31.5 mg./day 1-1-6)				NO DRUG
14	15	—		
—	—	282		
14	15	—	4/5	5/5
13	14	—		
15	16	—		
Primaquine (30 mg./day 1-1-6)				NO DRUG
—	—	450		
—	—	249		
—	—	429		
—	—	190		
—	—	311	0/10	10/10
—	—	284		
—	—	276		
—	—	253		
—	—	231		
—	—	218		
Primaquine (15 mg./day 1-1-6)				NO DRUG
—	—	239		
—	—	239		
—	—	239		
—	—	239		
—	—	260	2/10	10/10
—	—	260		
—	—	365		
19	20	—		
—	—	234		
15	16	—		
Primaquine (10 mg./day 1-1-6)				NO DRUG
12	13	—		
—	—	361		
14	15	—		
—	—	367		
—	—	239	3/10	10/10
—	—	230		
—	—	241		
—	—	238		
15	17	—		
—	—	335		

Primaquine was also given in doses of 10 and 15 mg. base daily (1-1-6) to two groups of ten men each (Table II). At these lower doses primaquine has a better capacity to protect against clinical malaria than has pamaquine at daily doses of 31.5 and 90 mg. base.

Intermittent (Semiweekly) Prophylactic Action.—In the preceding experiments primaquine administration on the day before inoculation, on the

TABLE III. THE EFFECTIVENESS OF EIGHT SEMIWEEKLY DOSES OF 30 MG. PRIMAQUINE BASE AS A "PROPHYLACTIC CURE"

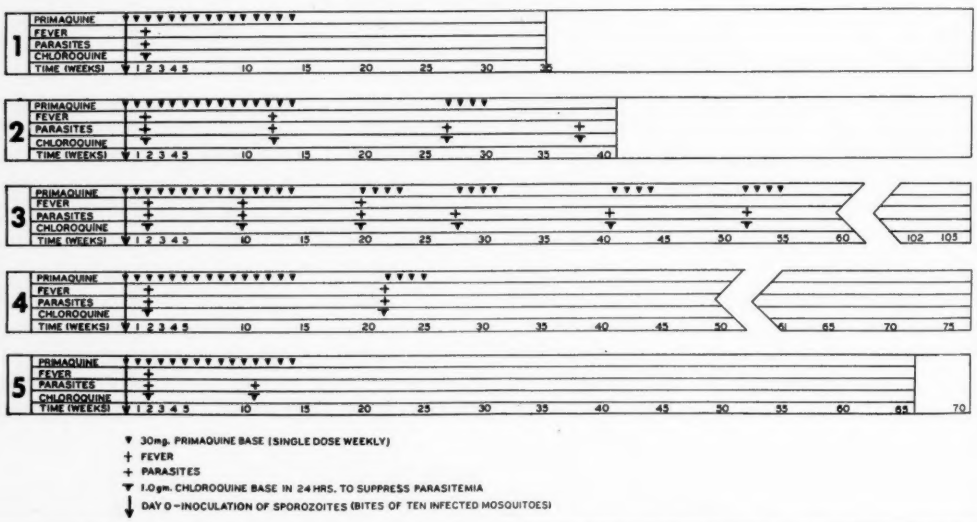


The first dose was given on the day of inoculation and continued for four weeks in testing against mosquito-induced *vivax* malaria (Chesson strain). All control volunteers, bitten by the same mosquitoes, developed overt malaria.

day of inoculation, and on each of the next six days (1-1-6) has been highly effective in the eradication of malaria but it is not of great interest in the practical management of *P. vivax.*

In order to devise a more practical program of drug administration, primaquine was given twice weekly for four weeks (Table III). In this program each volunteer received a single dose of 30 mg. primaquine base on Wednesday and Sunday of each week beginning on the day of infection and continuing for four weeks (a total of eight doses). This is exactly the same total dose as that given in the 1-1-6 experiments. Of twelve volunteers infected, only two developed patent malaria. The two treatment failures were given 1.0 Gm. chloroquine base during the first twenty-four hours of the clinical attack and this was followed by another course of eight semiweekly doses of 30 mg. primaquine base. They did not relapse after the second course of primaquine (Table III).

TABLE IV. THE EFFECTIVENESS OF PRIMAQUINE WHEN USED AS A "SUPPRESSIVE-CURE" IN CHESSON STRAIN VIVAX MALARIA

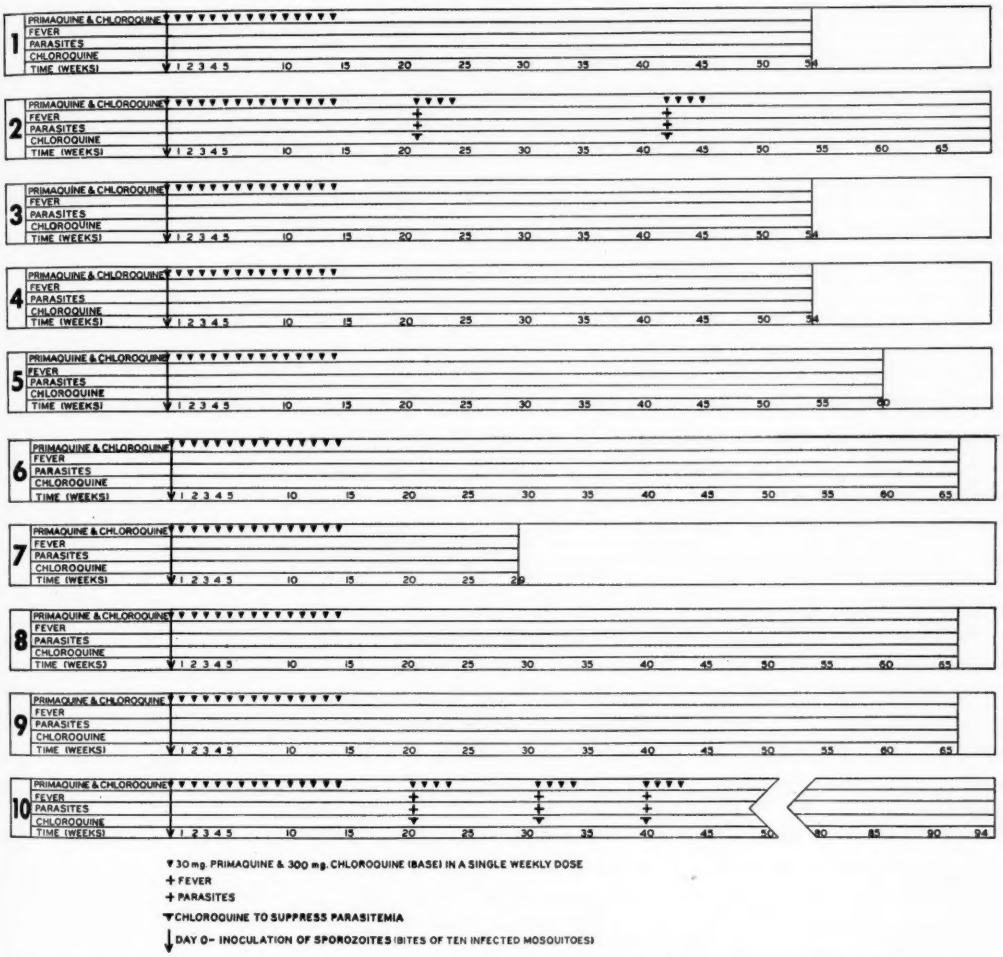


Drug was given in single doses once weekly for fifteen weeks with each clinical attack treated by 1.0 Gm. chloroquine base. All control volunteers, bitten by the same mosquitoes, developed overt malaria.

In order to discover whether subpatent infections developed trophozoites, two cases treated by this method were subinoculated into a second group of four nonimmune volunteers. These subinoculations were performed on day 9 and on day 15 following the sporozoite inoculation of the donor (Table III). All four subjects so inoculated remained negative for a period of thirty days,* at which time they were inoculated by mosquito bites which produced clinical malaria within fourteen days, thus demonstrating the volunteers' lack of immunity.

*Earle and associates found that twenty-one days was sufficient to demonstrate a failure of infection following intravenous inoculation of trophozoites.

TABLE V. THE EFFECT OF PRIMAQUINE USED AS A "SUPPRESSIVE-CURE" CONCURRENTLY WITH CHLOROQUINE IN CHESSON STRAIN VIVAX MALARIA



Both drugs were given in a single dose once weekly for fifteen weeks. All control volunteers, bitten by the same mosquitoes, developed overt malaria.

Intermittent (Weekly) Suppressive Cure.—Five other volunteers were treated by primaquine alone given in single weekly doses of 30 mg. base for a total of fifteen weeks (Table IV). Patency was not prevented by the first two doses which were given during the period normally required for the first clinical attack to develop. Consequently these cases had to be treated early in the program by 1.0 Gm. chloroquine base to control parasitemia. Two out of five cases receiving primaquine weekly for fifteen weeks (with administration of chloroquine each time patency occurred during the period) remained free of malaria twenty-one and fifty-two weeks, respectively, after their last dose of drug.

Eight out of ten volunteers receiving 30 mg. primaquine base concurrently with 300 mg. chloroquine base in a single weekly dose for fifteen weeks

remained free of parasites and asymptomatic during the course of therapy and for fifteen to fifty-one weeks after the last dose of drug (Table V).

Toxicity Studies.—In order to establish the toxicity of primaquine and chloroquine in single weekly doses, a group of sixty Negroes were given primaquine, 30 mg. base, and chloroquine, 300 mg. base, in a single dose once weekly for twelve months, and thirty-seven Negroes were given the same dose of drug for nine months. In none of these men was symptomatic toxicity elicited. Monthly analyses of blood and urine were also found to be normal.

DISCUSSION

It is apparent that there are several types of antimalarial drug. Each type is correlated with a different biologic stage of the parasite. For lack of precise information about the various forms of the parasite known as tissue or extra-erythrocytic stages, these forms have been divided into two main groups: (1) the pre-erythrocytic forms (or early tissue stages), and (2) the residual tissue stages which appear with and follow the production of the first brood of trophozoites (Fig. 1). The first brood of trophozoites serves as a convenient landmark dividing the two periods. This arbitrary division of tissue stages does not imply that either the pre-erythrocytic or the residual tissue forms are a single and unique form of the parasite or that we know the precise way in which the pre-erythrocytic tissue forms differ from the residual tissue forms.

The separation of tissue forms into two main groups leads to certain therapeutic concepts of practical significance. To point this out we have defined several special terms: 1. The term "causal prophylaxis" is used to indicate that a drug depends for its effect on an action against sporozoites or early tissue stages and not on its action against trophozoites or late tissue stages. 2. When drug action on early tissue stages is sufficient to prevent overt malaria but is not sufficient to prevent the development of late tissue stages so that medication must be continued beyond the usual prepatent period, we call this action "prophylactic-cure."

For "prophylactic-cure" the following conditions must be met: (a) trophozoites cannot be demonstrated during therapy, even by massive subinoculation; (b) the disease must not be cured by therapy during the usual incubation period; (c) continuation of therapy after the first nine days is necessary and sufficient to complete eradication of the tissue stages and achieve a radical cure. 3. When drug action in the incubation period is less effective than that producing a prophylactic cure, trophozoites appear. Some form of suppressive action, either that which may be inherent in the test drug itself, or, if that is lacking, a suppressive action from a supplementary drug, will be necessary to abort a clinical attack. While this suppressive action is operative the cumulative effect of continued treatment completely eradicates the tissue stages. This type of combined action we shall call "suppressive-cure."

For "suppressive-cure" the following conditions must be met: (a) trophozoites are formed although clinical symptoms are suppressed; (b) the

disease is not cured by therapy during the usual incubation period; (c) continuation of therapy after the first nine days is necessary and sufficient to complete eradication of tissue stages and achieve a radical cure.

The distinction among these three terms is based entirely on clinical-laboratory observations following therapeutic tests and cannot as yet be correlated with the anatomy or physiology of the parasite. The last two types of drug action have been produced by intermittent primaquine therapy over an extended period.

By far the easiest type of action to measure is "suppressive action" (action against trophozoites). Since illness from malaria is due almost entirely to the trophozoites, it is quickly apparent from the clinical response of the patient whether or not a drug is effective as a suppressive agent. Although it has been shown in numerous studies during World War II that suppressive drugs such as chloroquine, quinacrine, and quinine act only on the blood forms, this fact has not been generally appreciated.

So far, with the exception of chlorguanide and pyrimethamine, which appear to have a low order of prophylactic activity, the only drugs with a clear-cut action against the early tissue stages of vivax malaria have been members of the 8-aminoquinoline group, particularly those related to pamaquine. It is interesting to note that 8-aminoquinolines active against the tissue stage have a relatively poor action against the trophozoite and are all relatively rapidly excreted or degraded.*

Though it is interesting that true causal prophylaxis (1-1-6) can be demonstrated, the practical field advantage offered by a true causal prophylactic (1-1-6) without suppressive action is minimal. As for "intermittent prophylaxis" it is possible to say here only that primaquine given in doses of 30 mg. base twice weekly for four weeks to volunteers heavily infected with Southwest Pacific (Chesson strain) vivax malaria has protected ten out of twelve volunteers from all clinical manifestations of malaria, and ultimately has caused complete eradication of the infection. In addition, it was possible to to combine primaquine (30 mg. base) with chloroquine (300 mg. base) in fifteen single weekly doses to protect eight out of ten heavily infected volunteers from relapse after interruption of therapy. This we have called "suppressive cure." It would seem from this experience that semiweekly or weekly treatment with primaquine or with primaquine and chloroquine would be of greatest value in situations analogous to that faced by the American Army returning from Korea and that prophylactic therapy in endemic populations would be less important because of the chronic risk of reinfection.

CONCLUSIONS

Primaquine has been demonstrated to have a very powerful effect on tissue stages in the incubation period of vivax malaria. Daily doses of 15 mg. base begun on the day prior to inoculation and continued for the next seven days

*Unpublished observation of the authors.

prevent the ultimate development of clinical malaria. Some effect can be demonstrated with doses of 10 mg. daily for the same period.

When a dose of drug inadequate for "causal prophylaxis" is given during the incubation period and is continued into the clinical period and beyond that into the period of latency it has been shown that two effects are operative: (1) a delay in formation of trophozoites and eventual death of the tissue forms occurs so that no clinical attack is ever produced. This has been accomplished by giving 30 mg. primaquine base semiweekly for four weeks; (2) no delay in the formation of trophozoites is achieved, but eventual cure of the disease is accomplished by weekly doses of 30 mg. primaquine base with the addition of a suppressive agent in a high percentage of severe infections.

The practical implications of this last form of therapy are enhanced by the low order of toxicity produced by once weekly administration of primaquine, 30 mg. base, combined with chloroquine, 300 mg. base, to sixty Negroes for one year and thirty-seven Negroes for nine months. This form of therapy greatly reduces, if indeed it does not eliminate, the hazard of hemolytic anemia in Negroes, in contrast to the appreciable risk incurred in administration of daily doses of primaquine to Negroes (Hockwald and associates[5]). For this reason the intermittent method of therapy may be of considerable practical use in controlling malaria among military personnel, especially under conditions as they existed in Korea.

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THE HEMOLYTIC EFFECT OF PRIMAQUINE

IV. The Relationship of Cell Age to Hemolysis

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WHEN 30 mg. of primaquine is administered daily to individuals whose erythrocytes are primaquine-sensitive, an acute hemolytic anemia develops.[1] It was demonstrated in a previous study that this hemolytic process is self-limited.† If the administration of primaquine is continued in the same, or even in double, the dose which initially produced an acute hemolytic reaction, clinical evidence of hemolysis disappears and the hemoglobin gradually returns to normal. Cell survival studies have demonstrated that hemolysis ceases after about one-half of the original cell population is destroyed. It was shown that the remaining erythrocytes are not sensitive to the administration of primaquine even when they are transfused into a recipient who has never been given primaquine previously. It was also shown that, although about one-half of his original cell population is not hemolyzed, the sensitive individual retains the undiminished capacity to destroy about one-half of a new sensitive cell population transfused into him. It was thus established that the erythrocytes of sensitive individuals either develop tolerance to primaquine or form a heterogeneous population with respect to their sensitivity to the hemolytic action of primaquine.[2]

In the present study, the sensitivity of tagged young erythrocytes has been compared with that of tagged older erythrocytes to determine whether the population is heterogeneous in this respect.

METHODS

Fe^{59} was used to label red cells of a narrow age range. The available evidence indicates that this isotope is incorporated into newly formed, and not into mature, erythrocytes[3] and that it does not leave the red cell until it is destroyed.[4]

After a 500 ml. phlebotomy had been carried out, in order to increase iron utilization, a tracer dose of Fe^{59} Cl_3‡ was given intravenously to a primaquine-sensitive subject (Fig. 1). The radioactivity of blood samples was measured at intervals with a scintillation counter. For the sake of simplicity, a constant blood volume was assumed in calculating the percentage of the administered Fe^{59} which had been incorporated into the red cell

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This investigation was carried out under contract number DA-49-007-MD-20 between the Office of the Surgeon General, United States Army, and the Department of Medicine of the University of Chicago.

This work was further aided by the participation of Army Medical Officers assigned to the project by the Surgeon General of the United States Army.

The investigations reported in this paper would not have been possible except for the cooperation of the inmates and the administrative staff of Stateville Penitentiary.

Received for publication, May 28, 1954.

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†We do not wish to imply that it is safe, under usual clinical conditions, to continue administration of primaquine if acute hemolysis takes place. Under these conditions, the administration of primaquine must be stopped immediately.

‡The Fe^{59} Cl_3 used in this study was obtained from the Abbott Laboratories under an authorization from the Isotopes Division, U.S.A.E.C., Oak Ridge, Tenn.

mass.[4] The blood volume value used in these calculations had been obtained several months previously by a slight modification of the Na Cr^{51} O_4 method of Gray and Sterling.[5] Deviations of the blood volume from this value, such as increases in the blood volume due to infusion of saline solution (*A*, Fig. 1) or decreases in the blood volume due to acute hemolysis (*B*, Fig. 1), therefore caused slight deviations of the calculated from the actual percentage of the administered Fe^{59} in the red cell mass. Such deviations were small, however, and did not affect the interpretation of the results.

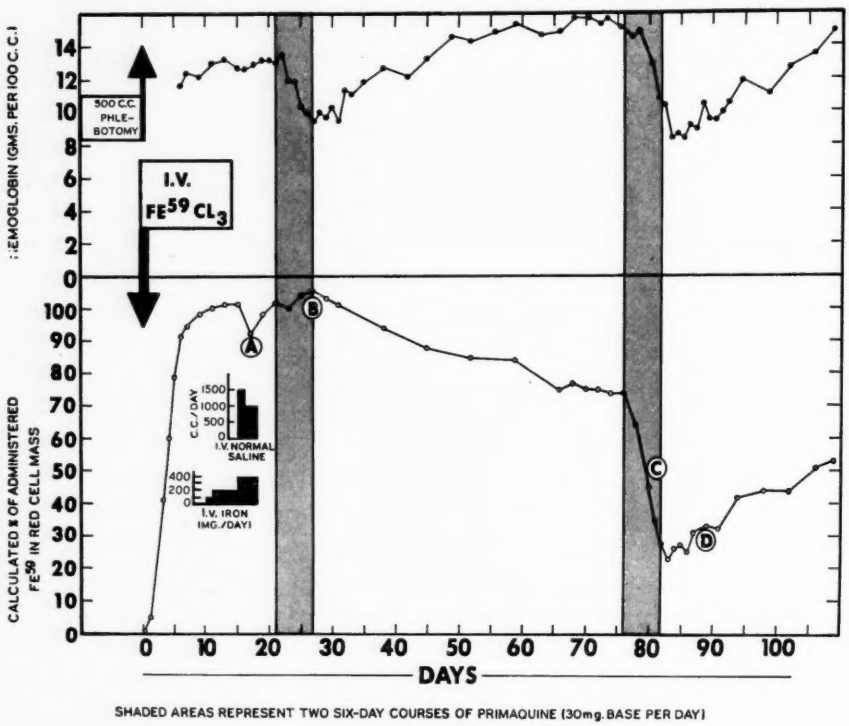

Fig. 1.—The effect of primaquine administration upon an Fe^{59} labeled red cell population with a narrow age range.

After the administered Fe^{59} had been almost completely utilized in the formation of red cells, 2.1 Gm. of nonradioactive saccharated iron oxide were given intravenously, to minimize the reutilization of any Fe^{59} released by hemolysis of labeled cells.[6] Incorporation of the Fe^{59} into erythrocytes was complete 13 days after its administration; the total age range of the labeled cells was therefore only 13 days. Two six-day courses of 30 mg. primaquine daily were given: first, 21 days after the administration of the Fe^{59} when the age of the labeled population varied from eight to 21 days; and second, 76 days after the administration of the Fe^{59} when the age of the labeled cell population varied from 63 to 76 days.

RESULTS

Fig. 1 presents the hemoglobin levels (upper curve) and the calculated percentage of Fe^{59} incorporated into the red cell mass (lower curve) during the course of this study. This figure demonstrates the completion of the uptake of the administered Fe^{59} by day 13. Subsequent changes in the amount of Fe^{59} in the blood (lower curve) represent almost exclusively the fate of erythrocytes produced during these 13 days.

The upper curve demonstrates the rapid fall in hemoglobin levels which occurred when each course of primaquine was administered. Although there was a fall of approximately 30 per cent in the hemoglobin level as a result of the first course of drug, there was no decline at all in the concentration of those cells aged 8 to 21 days, as represented by the concentration of Fe^{59} in the blood. Instead, there was actually a slight transient rise in the calculated percentage of the administered Fe^{59} in the red cell mass (*B*, lower curve). This rise is an artifact due to the reduction of blood volume which occurs during acute hemolysis (See Methods).

A gradual decrease in the calculated percentage of the administered dose of Fe^{59} in the red cell mass occurred between the two courses of primaquine. The greater portion of the fall was most probably due to a normal degree of random destruction of erythrocytes, but about 25 per cent of the decline could be accounted for in the frequent 10 ml. blood samples drawn for radioactivity determinations.

There was a fall of approximately 45 per cent in the hemoglobin level as a result of the second course of primaquine. The concentration of erythrocytes aged 63 to 76 days, as represented by the concentration of Fe^{59} in the blood, declined 66 per cent (*C*, lower curve) during the same period. The actual loss of these older cells must have exceeded this value, however, because some re-utilization of Fe^{59} released by hemolysis occurred, as evidenced by the post-hemolytic rise of the Fe^{59} concentration (*D*, lower curve).

DISCUSSION

An Fe^{59} labeled population of young erythrocytes (aged 8 to 21 days) was completely unaffected during a hemolytic reaction in which well over 30 per cent of the total red cell population was destroyed. Those cells which were hemolyzed must therefore have belonged to an older age group. That this was actually the case was also demonstrated directly: Fe^{59} labeled erythrocytes which had been in the circulation for slightly over one-half their normal life span were rapidly and preferentially destroyed when primaquine was administered.

Since destruction of erythrocytes in almost all hemolytic states heretofore studied is random with respect to red cell age,[7] the fact that hemolysis due to primaquine affects only older erythrocytes is particularly interesting. Heinz bodies formed in response to acetylphenylhydrazine[8] and sulfanilamide[9] have been shown to spare the reticulocytes. Thus one similarity between red cell damage by these agents and by primaquine immediately becomes apparent. Other evidence has also accumulated indicating that a close relationship exists between the hemolytic anemia induced in sensitive subjects by primaquine and that produced by certain aniline derivatives, such as sulfanilamide and phenylhydrazine. It was shown that Heinz bodies, which are characteristically produced in erythrocytes by these aniline derivatives, are also produced in the course of the primaquine-induced hemolytic reaction.[10] Furthermore, Dern and associates[11] have demonstrated that primaquine-sensitive erythrocytes are also unusually sensitive to the hemolytic action of the same aniline derivatives.

Sensitivity to the hemolytic action of primaquine is due to an intrinsic

"defect" of the red cell.[1] The nature of the defect has not been elucidated, in spite of detailed studies of the erythrocytes by conventional hematological methods.[10] The activity of some of the enzyme systems in the red cell are believed to decrease as they age.[12] The fact that only older erythrocytes are sensitive to primaquine suggests that a deficiency in one of the many enzyme systems of the red cell may be involved in the susceptibility of sensitive cells to the hemolytic action of primaquine. Studies of P^{32} uptake and of catalase activity have revealed no significant differences between primaquine-sensitive and nonsensitive erythrocytes.[13] Further investigations of the enzymes of primaquine-sensitive and nonsensitive erythrocytes are being carried out. It is hoped that these studies will reveal the difference between sensitive and nonsensitive erythrocytes, and in so doing, will help elucidate the mechanisms of the hemolytic action of this entire group of hemolytic compounds.

SUMMARY

Erythrocytes of uniform age from a primaquine-sensitive individual were labeled with Fe^{59}. When the labeled erythrocytes were 8 to 21 days old, they were insensitive to the hemolytic action of primaquine; 55 days later the same cells were rapidly destroyed when another course of primaquine was administered. This finding further strengthens the relationship between hemolysis by primaquine and hemolysis by certain aniline derivatives. The increased sensitivity of older cells is believed to suggest that a deficiency in one of the red cell enzymes may play a role in primaquine sensitivity.

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NONSPECIFIC REACTIONS WITH THE MIDDLEBROOK-DUBOS TEST FOR TUBERCULOSIS ANTIBODIES

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THE hemagglutination test for tuberculosis[1] and its hemolytic modification[2] have yielded a high incidence of positive reactions with sera of patients who had no evidence of tuberculous disease. In some instances these "false positive" tests were apparently anamnestic responses after tuberculin skin tests.[3, 4] In others the cause was not investigated.[3, 5, 6, 7] Thus, the clinical value of the test has remained limited. Cross reactions with nontuberculous antibodies were anticipated and investigated by Middlebrook and Dubos,[1] but none were found in various immune rabbit sera. Rothbard,[8] however, noted such reactions with staphylococcus immune serum.

It is obviously difficult to obtain from patients valid data of previous exposure to infections which may have given rise to antibody formation. Experimental studies were, therefore, undertaken to explore the factors other than tuberculous infection that led to positive reactions in the Middlebrook test, and the possibility of differentiating cross reactions.

MATERIALS AND METHODS

Animals.—White rabbits of 2,000 to 2,500 grams were used. A Middlebrook-Dubos test was done on the sera of all of them before immunization or other procedures were carried out.

Bacterial Suspensions and Adjuvants.—For immunization, suspensions of a twenty-four-hour growth of *Staphylococcus aureus* or beta hemolytic streptococcus were obtained and autoclaved. Suspensions containing 11 billion microorganisms of the staphylococci, and 13 billion organisms of the streptococci per milliliter (counted in a Petroff-Hausser chamber) were prepared and diluted as desired. For immunization of animals, suspensions containing one billion organisms per milliliter were used. Arlacel* and heavy mineral oil, 1:2 parts, were mixed in an electric blendor, and this was employed as the adjuvant in the first group of rabbits. In the second group, an adjuvant made up with Aquaphor, as recommended by Wood,[10] was inoculated. All adjuvant injections were given intracutaneously.

Middlebrook-Dubos Test.—The procedures, as outlined by the authors,[1, 2] were closely followed. All sera were kept in a refrigerator for at least twenty-four hours before use. It was noted that different lots of Lederle Old Tuberculin (O.T.) 4 X differed in their sensitizing strength and that they were not comparable as far as cross reactions were concerned. To assure uniformity, Lot 2725-33† was used throughout these experiments.

Preparation of Polysaccharide.—Pneumococcus polysaccharide was prepared from Type II pneumococcus, as recommended by Anderson and McCarty.[9] Somatic polysaccharide

From the Veterans Administration Hospital, Oakland, Calif.

This investigation was supported by grants from the Committee on Medical Research of the American Trudeau Society, Medical Section of the National Tuberculosis Association, and from the California Tuberculosis and Health Association.

Received for publication, May 28, 1954.

*Arlacel A, Batch 3618, obtained from the Atlas Powder Co., Wilmington, Del.

†Tuberculin was generously supplied by Dr. H. D. Piersma, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

from staphylococci and streptococci was obtained by lysing the washed, ground, and dried bacterial cells with Formamide (10 Gm. of bacilli to 150 ml. Formamide), and then proceeding as with the pneumococci. Solutions containing 1 mg. polysaccharide per milliliter saline were used. The same strain of microorganisms was employed for immunization, preparation of polysaccharide, and absorption experiments.

Antiserum to Cx-Reactive Protein.—This was kindly supplied by Dr. H. F. Wood of the Rockefeller Institute. Precipitin tests were set up according to this author.[10]

RESULTS

Inoculations Eliciting Nontuberculous Antibodies.—One rabbit (No. 180) was inoculated intravenously with three injections of staphylococcus suspension at weekly intervals. The serum obtained on the fourth day after the last injection showed a titer of 1:256 in the Middlebrook-Dubos test. In another rabbit (No. 238) a titer of 1:32 was obtained two weeks after the second of two intracutaneous injections. The lower titer after intracutaneous immunization is in accord with common experience. The rabbit immunized with streptococci (No. 191) also received three intravenous injections at weekly intervals, and seven days after the last inoculation had a titer of 1:256.

As a control, tuberculous immune serum was obtained from a rabbit inoculated with 0.4 mg. of Ravenel bovine tubercle bacilli. This serum obtained on the fourteenth day after inoculation showed a titer of 1:128.

In an early experiment it was observed that the serum of a rabbit injected with Arlacel adjuvant gave a positive reaction in the Middlebrook-Dubos test. This finding was confirmed in other rabbits which had received this adjuvant or one with Aquaphor. The interval at which these antibodies appeared and their chronologic relation to the Cx-reacting protein is shown in Table I.

TABLE I. CORRELATION BETWEEN CX-PROTEIN RESPONSE AND DEVELOPMENT OF ANTIBODY REACTING IN MIDDLEBROOK-DUBOS TEST

RABBIT NO.	ADJUVANT	INTERVAL IN DAYS AFTER LAST INJECTION														
		1			2			3			5			7		
		M*	CX†	P‡	M	CX	P	M	CX	P	M	CX	P	M	CX	P
232	Arlacel	+1:8	+	–	+1:8	+	–	+1:4	+	–	0	0	0	–	+	–
234	Arlacel	+1:8	+	–	–	–	–	Died								
243	Arlacel	–	–	–	–	–	–	+1:4	–	–	0	0	0	+1:4	–	–
251	Aquaphor	–	–	0	–	–	0	0	0	0	+1:4	+	0			
240	Aquaphor	–	–	0	–	–	0	–	+	0						

*M = Antibody reacting in Middlebrook-Dubos test.
†Cx = Precipitin test with antiserum to Cx-Reactive Protein.
‡P = Precipitin test with Pneumococcus II Polysaccharide.
+ = Positive.
– = Negative.
0 = Not done.

Differentiation of Cross Reactions.—Middlebrook and Dubos[1] stated that the tuberculous antibodies reacting in their test could be inhibited by incubating the serum with small amounts of tuberculin. This technique was applied to tuberculous and to other immune sera. As high a dilution as 10^{-6} of O.T. was capable of completely inhibiting tuberculous immune serum in the Middlebrook-Dubos test. In contrast, the streptococcus immune rabbit serum could be inhibited only by a concentration of 1:100, and that of staphylococcus im-

mune rabbit serum by a 1:40 dilution of the identical O.T. The activity of the serum from rabbits inoculated with adjuvant was inhibited with a 10^{-3} dilution of O.T., further dilutions not being tested. Hence, by the technique of inhibition with O.T., two groups of nonspecifically reacting sera could be differentiated.

It recently has been shown[11, 12] that a variety of agents, including the individual constituents of adjuvant, are able to induce the production of Cx-reacting protein in rabbits. This protein characteristically appeared soon after the administration of the agent. The antibody reacting with O.T. sensitized red cells could be found within twenty-four hours after the injection of adjuvant. The time relationship of the two shown in Table I suggested that the acute phase protein was reacting also in the Middlebrook-Dubos test. Differentiation of the two reactions by removal of calcium ions[13] proved not feasible, since this also prevented the reaction with the O.T. sensitized cells.

It was noted that the staphylococcus and streptococcus, as well as one of the tuberculous immune sera (Rabbit No. 237), gave a positive reaction with the antiserum to Cx-reactive protein and with somatic polysaccharide from pneumococcus Type II. Their different responses to inhibition by O.T., and the absence of acute phase protein in other tuberculous immune rabbit sera, led to an attempt to resolve the overlapping antibody activities by consecutive absorption or precipitation of antibodies. Precipitin tests were done on representative sera with the homologous somatic polysaccharide and with Cx-protein antiserum, after the sera had been absorbed once with O.T. sensitized cells. The absorbed immune sera retained their ability to react with the homologous polysaccharide as well as with the Cx-protein antiserum. The sera of three rabbits (No. 232, 240, and 251) injected with adjuvant lacked the acute phase protein after the absorption procedure. All of these had reacted with the Cx-protein antiserum, and two of them had given a positive Middlebrook-Dubos test (Table I). This experiment was repeated, subjecting the immune sera to three subsequent presumably exhaustive absorptions with equal amounts of a 2 per cent suspension of O.T. sensitized red cells. This removed the Cx-reacting protein as evidenced by negative tests with the specific antiserum. The repeated absorption eliminated also the antibody precipitating with the homologous polysaccharide. Failure to react was not due to the dilution, since similarly diluted but unabsorbed sera retained their reactivity.

In the next experiment the sera were first treated with either one of the somatic polysaccharides. The precipitate which formed was separated by centrifugation, and the sera then employed for agglutination tests with O.T. sensitized red cells. After removal of the red cells by centrifugation, Cx-protein antiserum was added. The results are shown in Table II. The absorption with polysaccharide did not remove the capacity of the serum to react in the Middlebrook-Dubos test, nor did both procedures together prevent formation of a precipitate with the Cx-protein antiserum. This procedure was repeated using double the amount of polysaccharide (2 mg. per milliter) with the same results except for the staphylococcus immune serum No. 238 where both anti-

body activities had disappeared. These results furnished more suggestive evidence that at least two different antibodies are present in the staphylococcus and streptococcus immune sera.

TABLE II. REACTIONS OF RABBIT SERA IN MIDDLEBROOK-DUBOS TEST AND WITH CX-ANTISERUM BEFORE AND AFTER PRECIPITATION WITH BACTERIAL POLYSACCHARIDES

RABBIT NO.	237	180	238	191	232	240	251
INOCULATED WITH	TB	STAPH.	STAPH.	STREP.	ADJ.*	ADJ.	ADJ.
Before precipitation:							1:4
Titer in Middlebrook-Dubos test	1:64	1:64	1:32	1:512	1:8	–	
Reaction with Cx-antiserum	+	+	+	+	+	+	+
Middlebrook-Dubos test after precipitation with:							
Staph. Polysaccharide	+	+	+	+	+	0	–
Strep. Polysaccharide	+	+	+	+	+	0	–
Reaction of supernatant serum with Cx-antiserum	+	+	+	+	–	0	–

*Adj. = Adjuvant.
+ = Positive.
– = Negative.
0 = Not done.

A further attempt at differentiating antibodies was made by absorbing the sera with suspensions of whole bacilli (Table III). The reactivity of the tuberculous, the streptococcus immune, and the adjuvant sera was left intact by this procedure; that of the staphylococcus immune sera was removed by the concentrated suspension of the homologous microorganisms. The reactivity with the Cx-antiserum was preserved in the tuberculous and streptococcus immune sera.

TABLE III. REACTIONS OF RABBIT SERA IN MIDDLEBROOK-DUBOS TEST AND WITH CX-PROTEIN ANTISERUM BEFORE AND AFTER ABSORPTION WITH BACTERIAL SUSPENSION

RABBIT NO.	237	180	238	191	251
INOCULATED WITH	TB	STAPH.	STAPH.	STREP.	ADJUVANT
Before absorption:					
Titer in Middlebrook-Dubos test	1:64	1:128	1:8	1:32	1:4
Reaction with Cx-antiserum	+	+	+	+	+
Middlebrook-Dubos test after absorption with:					
Staphylococci					
1:100	+	+	–	+	+
1:5,000	+	–	±	+	+
Streptococci					
1:100	+	+	–	+	+
1:5,000	+	+	±	+	+
Reaction with Cx-antiserum after absorption with:					
Staphylococci					
1:100	+	–	–	+	±
Streptococci					
1:100	±	±	–	+	±

+ = Positive.
– = Negative.
± = Doubtful.

Another approach to distinguishing the different antibodies reacting in the Middlebrook-Dubos test would be a uniform sensitizing agent. A purified fraction of O.T. (Fraction PS)* has been prepared recently by Dr. Middlebrook. This was employed for sensitizing red cells. The tuberculous, staphylococcus, and streptococcus immune sera gave reactions of a titer similar to that obtained with O.T. sensitized cells, while the serum of an adjuvant-injected (No. 251) reacted in a twofold higher dilution. Ascending chromatograms revealed that PS contained four ninhydrin-positive fractions with distinct, O.T. several fractions with confluent, boundaries. Both O.T. and PS showed three silver-reducing fractions, one of those of the O.T. moving farther from the origin than in the chromatogram of the PS. PS, therefore, could not be considered as a homogenous sensitizing agent. Attempts to utilize individual fractions eluted from multiple paper chromatograms for sensitizing red cells were unsuccessful, since the eluted material with or without dialysis caused lysis of red cells.

DISCUSSION

Immunization of rabbits with staphylococci or streptococci causes them to produce circulating antibodies of a high titer that react in the Middlebrook-Dubos test. Evidence has been presented by other authors[1, 14] that the polysaccharides, of the substances found in tuberculin, are adsorbed into untreated red cells and act as sensitizing agent. Thus, the antibodies tested by the Middlebrook-Dubos procedure are assumed to be of the antipolysaccharide type. When the staphylococcus and streptococcus immune sera were exposed to homologous bacterial polysaccharide, and the antigen-antibody complex which formed was removed, the sera still contained antibodies capable of reacting in the Middlebrook-Dubos test. This indicates that at least two antibodies are present in these immune sera.

The capacity of the staphylococcus and streptococcus immune sera to react in the Middlebrook-Dubos test, in contrast to that of tuberculous immune serum, is not inhibited by a 10^{-3} dilution of O.T. Absorption with a suspension of homologous microorganisms removed the antibody activity of the staphylococcus immune, but not that of the streptococcus immune serum. Thus, the antibodies developing after these two infections may be differentiated from each other, and from those in the tuberculous immune serum by an albeit cumbersome procedure.

The coincidence of appearance of the Cx-reacting protein and of reactivity in the Middlebrook-Dubos test in the sera of rabbits inoculated with adjuvant, the similarity of absorption and failure to be absorbed by various procedures (Tables II and III) are suggestive evidence that the Cx-reacting protein is the one in these sera reacting with the O.T. sensitized cells. Its activity is indistinguishable from the tuberculous antibody by the means employed. Rabbit Cx-protein is analogous to human C-protein.[10] The latter often occurs after an injury,[15, 16] and differentiation of its reaction from that of the tuberculous antibody is essential to the clinical validity of the Middlebrook-Dubos test.

*Kindly supplied by Dr. G. Middlebrook.

SUMMARY

Antibodies reacting at a high titer in the Middlebrook-Dubos test were found in sera of rabbits immunized with either staphylococci or streptococci.

Antibodies of a low titer were noted in sera of rabbits inoculated with adjuvant. This antibody activity is associated with the appearance of Cx-reacting protein.

Antibody activity of staphylococcus and streptococcus immune sera can be distinguished from that of tuberculous immune serum by the procedure reported.

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EFFECT OF ANTIBIOTICS ON ORGANISMS USED IN CERTAIN MICROBIOLOGIC ASSAYS OF ESSENTIAL NUTRIENTS

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IN THE course of metabolic studies on the effect of antibiotics on the urinary and fecal excretion of certain nutrients in man,[1] it was necessary to determine whether the presence of antibiotic in the materials to be assayed would affect the results of assays, especially those utilizing microorganisms for the determination of amino acids and vitamins. Results obtained by these methods could conceivably be influenced either by inhibition of growth of the assay organism by the antibiotics contained in the material to be assayed, perhaps through specific antagonism of one or another of the essential nutrients in the medium, or, on the contrary, by enhancement of the growth of the assay organism by the antibiotics or their derivatives, should these replace any essential nutrient required for growth of that organism. This report presents data concerning the sensitivity of microbiologic assay organisms to certain antibiotics and the effect of substituting some of the latter for various nutrients required for the growth of these organisms. Inasmuch as most of the emphasis in the studies on nutrition concerned the effects of chlortetracycline (Aureomycin†), particular attention was paid to this antibiotic in the present study.

MATERIALS AND METHODS

The following are the organisms studied and the microbiologic assay media and methods in which each was employed. *Streptococcus faecalis* (ATCC 9790) was used for the assay of arginine, histidine, isoleucine, leucine, lysine, methionine, and valine in the synthetic amino acid medium (AAM) of Stokes and associates.[2] *Lactobacillus casei* (ATCC 9595) was used in the same method for measuring phenylalanine[3] and *L. delbrueckii* LD5‡ was also employed in the same method for the assay of tyrosine.[3] *L. casei* (ATCC

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Supported, in part, by a grant from the United States Public Health Service and, in part, by a grant from the Surgeon General, Department of the Army, under the sponsorship of the Commission on Liver Diseases, Armed Forces Epidemiological Board.

Received for publication, May 29, 1954.

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†Since the generic name chlortetracycline was introduced after these studies were completed, this term appears in the text and tables but the original designation Aureomycin (now the trademark of Lederle Laboratories Division, American Cyanamid Co., for chlortetracycline) is used in the figures.

‡This organism, originally obtained from Dr. Stokes of Merck and Company, Inc., is the same as *L. casei* (ATCC 9595) according to personal communications from Dr. Ruth E. Gordon (Nov. 6, 1948) and from Dr. Freeman A. Weiss (March 8, 1954), Curators of the American Type Culture Collection. Since both strains were maintained separately and used for different purposes in this laboratory, the results obtained with each of them will be presented here.

TABLE I. ANTIBIOTIC SENSITIVITY OF SOME MICROBIOLOGIC ASSAY ORGANISMS AS DETERMINED IN VARIOUS MEDIA

ORGANISM	MEDIUM AND METHODS*	MINIMUM COMPLETE INHIBITING CONCENTRATION, MICROGRAMS PER MILLILITER								
		PENICILLIN	CHLORTETRACYCLINE	OXYTETRACYCLINE	NEOMYCIN	CHLORAMPHENICOL	STREPTOMYCIN	BACITRACIN	POLYMYXIN	ERYTHROMYCIN
S. faecalis (ATCC 9790)	HIA (12)	8.0	6.3	6.3	>400	12.5	400	0.5	>400	0.2
	BHIB (12)	8.0	6.3	0.8	>400	12.5	>400	0.5	>400	0.02
	AAM (2)	1.0	3.1	12.5	25	25	50	8.0	>800	1.6
L. casei (ATCC 9595)	HIA (12)	0.5	25	12.5	25	12.5	12.5	0.25	400	0.04
	BHIB (12)	0.5	3.1	1.6	12.5	6.3	12.5	0.5	400	0.01
	AAM (3)	0.5	6.3	50	3.1	25	3.1	>8.0	25	1.6
L. delbrueckii LD5‖ (ATCC 9595)	HIA (12)	0.5	25	12.5	12.5	12.5	12.5	0.13	400	0.1
	BHIB (12)	0.5	3.1	3.1	6.3	6.3	25	0.13	12.5†	0.02
	AAM (3)	1.0	12.5	50	3.1	25	12.5	>8.0	800	0.8
L. casei MNL (ATCC 7469)	HIA (12)	0.5	12.5	12.5	25	12.5	12.5	0.25	400	0.1
	BHIB (12)	0.5	3.1	3.1	25	6.3	25	0.5	400	0.01
	RM (5)	0.5	3.1	>50	12.5	25	100	>8.0	>800	1.6
	FAM (4)	0.25	12.5	25	12.5	12.5	6.3	>8.0	50	0.8
L. citrovorum (ATCC 8081)	HIA (12)	2.0	100	200	3.1	12.5	25	1.0	12.5	0.1
	BHIB (12)	1.0	100	100	12.5	6.3	50	2.0	12.5	0.02
	CFM (6, 7)	2.0	200	200	>100	12.5	200	>8.0	800	3.2
L. arabinosus 17-5 (ATCC 8014)	HIA (12)	16.0	100	100	3.1	6.3	12.5	1.0	25	0.1
	BHIB (12)	2.0	25	25	3.1	3.1	25	2.0	25	0.01
	NAM (8, 9)	1.0	100	200	25	12.5	100	>8.0	50	0.8
S. carlsbergensis (ATCC 4228)	PM (10, 11)‡	>64	>800	>800	>800	>100	>800	>8.0	100	>200
Streptococcus 98§	HIA (12)	0.01	1.6	1.6				0.03		0.1
	BHIB (12)	0.02	1.6	0.8				0.02		0.1
Kl. pneumoniae T§	HIA (12)				1.6		3.1			
	BHIB (12)				3.1	6.3	1.6			
E. coli, McLeod§	HIA (12)								1.6	
	BHIB (12)								0.1	

*For explanation of abbreviations see Materials and Methods. Numbers in parentheses indicate references to methods in which media are used.

†Poor growth in control.

‡This organism failed to grow adequately on agar or in broth and therefore was not tested in these media.

§Organisms used as controls in antibiotic assays.

‖ See ‡ footnote on page 449.

7469) was employed for the determination of folic acid by the method (FAM) of Tepley and Elvehjem[4] and for the assay of riboflavin by the method (RM) of Snell and Strong.[5] *Leuconostoc citrovorum* (ATCC 8081) was utilized for assay of citrovorum factor by the method (CFM) of Sauberlich and Baumann.[6, 7] *Lactobacillus arabinosus* 17-5 (ATCC 8014) was employed to measure nicotinic acid by the method (NAM) of Snell and Wright[8] as modified by Snell[9] and *Saccharomyces carlsbergensis* (ATCC 4228) was used in the assay of pyridoxine by the method (PM) of Atkin and colleagues [10] as modified by Rabinowitz and Snell.[11] These media were all freshly prepared from amino acids, vitamins, and salts.

Three methods were used for determining the sensitivity of these organisms to various antibiotics: (1) a *plate dilution method* employing heart infusion agar (HIA)[12]; (2) a *tube-dilution method,* using brain-heart infusion broth (BHIB)[12]; and (3) a method similar to the latter but using the appropriate microbiologic assay medium (as noted previously) and incorporating the optimum amounts of the ingredients for each of the test organisms. Incubation was carried out at 37° C. for all tests except those with *S. carlsbergensis* in PM. Readings were made after 48 hours in the tests on agar or in broth, after 60 hours in those done in AAM, and at 72 hours when the vitamin assay media (RM, FAM, CFM, and NAM) were used. In the tests with *S. carlsbergensis* in PM, readings were made after incubation for 16 hours at room temperature. The end points in the methods utilizing heart infusion agar and beef heart infusion broth were based on observations of visible growth of the organisms,[12] whereas in the tests utilizing the various assay media other than PM, the production of acid or failure to do so were used as indications of growth and inhibition, respectively. Titrations of acidity were done with appropriate concentrations of NaOH, using bromthymol blue as indicator. The growth of *S. carlsbergensis* in PM was measured turbidimetrically.

The following antibiotics were used: penicillin, obtained commercially as crystalline potassium penicillin G; streptomycin, obtained commercially as the crystalline sulfate, chlortetracycline (Aureomycin), supplied as the crystalline hydrochloride by Dr. Stanton M. Hardy of Lederle Laboratories Division, American Cyanimid Company; oxytetracycline (Terramycin), furnished as the crystalline hydrochloride by Dr. Gladys L. Hobby of Chas. Pfizer & Co., Inc.; chloramphenicol (Chloromycetin) supplied by Dr. E. A. Sharp of Parke, Davis & Company; neomycin sulfate made available by Dr. H. F. Hailman of Upjohn Co.; bacitracin (assayed at 41.3 units per milligram) supplied by Dr. Lawrence W. Smith of Commercial Solvents Corp.; polymyxin B sulfate (Aerosporin) furnished by Dr. Donald F. Searle of Burroughs Wellcome & Co. (U.S.A.), Inc.; erythromycin (Ilotycin) obtained from Dr. J. W. Smith of Eli Lilly & Company.

Most of the amino acids and vitamins were obtained from Merck and Company, Inc.; folic acid (supplied as synthetic pteroylglutamic acid), citrovorum factor, and Aminopterin were furnished by Lederle Laboratories Division, American Cyanamid Company through the courtesy of Drs. T. H. Jukes and James M. Ruegsegger.

RESULTS

Sensitivity of Microbiologic Test Organisms to Antibiotics.—The results of the tests for the antibiotic sensitivity of some microorganisms used in the microbiologic assay of essential nutrients as carried out in various media are listed in Table I. The sensitivity of the organisms used for controls in antibiotic assays in this laboratory are given for comparison; since they grew poorly in the microbiologic assay media, however, the tests for their sensitivity to antibiotics in these media are not recorded. There appears to be no difference in the inhibitory action of chlortetracycline, penicillin, and chloramphenicol whether tested on the usual bacteriologic media or in the special assay media. On the other hand, significantly larger concentrations of bacitracin and erythromycin were required to completely inhibit growth of the test

§Organisms used as controls in antibiotic assays.
‖ See ‡ footnote on page 449.

TABLE II. EFFECT OF AUTOCLAVING ON THE ACTIVITY OF THREE ANTIBIOTICS AGAINST MICROBIOLOGIC ASSAY ORGANISMS

STRAIN	AUTOCLAVED*	ANTIBIOTIC	TITRATABLE ACIDITY, MILLILITERS OF 0.05 NORMAL NaOH† — CONCENTRATION OF ANTIBIOTIC, MICROGRAMS PER MILLILITER										
			0	200	100	50	25	12.5	6.3	3.1	1.6	0.8	0.4
S. faecalis	Yes	Chlortetracycline	7.6	4.7	6.1	6.2	6.4	6.8	6.9	7.2	7.1	7.5	7.9
S. faecalis	No	Chlortetracycline	7.8	1.5	1.1	1.3	1.1	1.2	1.0	1.1	9.6	10.5	5.9
S. faecalis	Yes	Oxytetracycline	7.7	6.5	6.5	6.3	6.8	6.5	7.0	6.9	7.0	6.9	7.1
S. faecalis	No	Oxytetracycline	6.9	0.9	1.0	0.8	0.9	0.8	1.0	1.1	0.8	4.8	5.5
S. faecalis	Yes	Chloramphenicol	7.4	0.6	0.5	0.7	0.6	3.4	5.3	6.8	6.8	7.2	7.1
S. faecalis	No	Chloramphenicol	7.0	0.7	1.0	0.7	0.9	2.0	5.3	6.5	6.4	7.3	7.0
L. casei, 9595	Yes	Chlortetracycline	5.6	5.4	5.7	6.0	5.6	5.5	5.4	5.6	5.8	6.1	6.1
L. delbrueckii, LD5	Yes	Chlortetracycline	6.0	5.1	5.3	5.6	5.7	6.4	5.0	6.0	5.6	5.9	5.5
L. casei, 9595	Yes	Oxytetracycline	5.6	5.2	5.4	5.8	5.2	5.7	5.7	5.2	5.3	5.6	6.0
L. delbrueckii, LD5	Yes	Oxytetracycline	5.3	5.6	5.8	5.4	5.5	6.1	5.2	5.5	5.6	5.8	5.2
L. casei, 9595	Yes	Chloramphenicol	5.6	0.4	0.4	0.3	0.6	2.4	4.6	4.9	5.6	5.4	5.6
L. delbrueckii, LD5	Yes	Chloramphenicol	5.6	0.3	0.3	0.3	0.5	2.7	4.8	5.5	5.1	5.7	5.9
None (Drug control)	Yes	Chlortetracycline	0.5	0.8	0.7	0.9	0.7	0.5	0.6	0.5	0.5	0.5	0.6
None (Drug control)	No	Chlortetracycline	0.5	0.9	1.1	0.9	0.9	0.8	1.1	1.0	1.0	1.2	1.4
None (Drug control)	Yes	Oxytetracycline	0.5	1.5	1.0	0.8	0.7	0.6	0.6	0.7	0.7	0.5	0.5
None (Drug control)	No	Oxytetracycline	0.5	1.3	1.0	0.7	0.9	0.9	0.9	0.9	0.8	0.9	0.8
None (Drug control)	Yes	Chloramphenicol	0.5	0.6	0.6	0.5	0.5	0.5	0.5	0.6	0.5	0.5	0.6
None (Drug control)	No	Chloramphenicol	0.5	0.9	0.7	0.9	0.9	0.9	1.1	1.1	0.9	1.2	0.9

*Fifteen pounds for 15 minutes after antibiotics added but before the organisms were inoculated.

†After incubation for 60 hours at 37° C. – complete synthetic amino acid medium.[2,3]

strains in the assay media as compared with those required when they were grown on agar or in broth containing these antibiotics. The results with oxytetracycline, neomycin, and streptomycin in the different media were variable. In the case of *L. citrovorum,* when it was grown in its complete specific medium (CFM), the minimum complete inhibiting concentrations of the antibiotics other than penicillin, the tetracyclines, and chloramphenicol, were greater than when this organism was tested on agar or in broth.

Effect of Autoclaving on Antibiotic Activity.—Under the usual conditions of the microbiologic assays, the antibiotics present in materials to be assayed for nutrients are autoclaved with the media prior to inoculating the organisms. The effect of the autoclaving on the activity of three antibiotics was tested using concentrations of antibiotics such that, when unautoclaved, the highest

was greater and the lowest was less than the minimum complete inhibiting concentration required for the test organisms, as shown in Table I. The results are shown in Table II. Using *S. faecalis* as the test organism in the complete synthetic medium (AAM) at pH 7.4, autoclaved chlortetracycline and oxytetracycline, in the three concentrations used, failed to inhibit the growth of the organism, as evidenced by the fact that the amount of final titratable acidity was similar to that of the control cultures in the complete medium containing no antibiotic; the only exception was in the tube containing the highest concentration of chlortetracycline (200 μg per milliliter) which showed somewhat reduced titratable acidity, indicating partial growth.* The unautoclaved chlortetracycline and oxytetracycline, on the other hand, inhibited the growth of *S. faecalis* in concentrations of 3.1 and 1.6 μg per milliliter, respectively, and in all greater concentrations. Chloramphenicol, both autoclaved and unautoclaved, produced partial inhibition of growth of *S. faecalis* in a concentration of 12.5 μg per milliliter and complete inhibition in greater concentrations. These observations using *S. faecalis* show that the antibiotic activity of chlortetracycline and oxytetracycline, in the concentrations used, was destroyed by autoclaving, except for some slight residual activity of chlortetracycline in the tube containing 200 μg per milliliter. The activity of chloramphenicol, on the other hand, was totally unaffected by the autoclaving under the same conditions.

The effect of autoclaved antibiotics on the growth of *L. casei* 9595 and *L. delbrueckii* LD5 in the complete synthetic medium AAM is also shown in Table II. Neither chlortetracycline nor oxytetracycline, in the range of concentrations used, inhibited the growth of the organisms when the antibiotics were autoclaved before the organisms were inoculated. Autoclaved chloramphenicol, on the other hand, produced partial inhibition of both strains in a concentration of 12.5 μg per milliliter and complete inhibition in all higher concentrations.

Replacement of Amino Acids by Antibiotics.—An attempt was made to determine whether antibiotics could replace certain "essential" amino acids in supporting the growth of some of the microbiologic assay organisms. The antibiotics tested were chlortetracycline, oxytetracycline, and chloramphenicol. The complete synthetic medium, AAM, at pH 7.4, was used as the control and each of the three antibiotics, in concentrations of 100, 10, and 1 μg per milliliter was substituted, in turn, for each one of eleven amino acids essential for the growth of *S. faecalis* and *L. casei.* The media were autoclaved after addition of the antibiotics and before inoculation of the organisms.

The results are shown in Table III. In the absence of any one of the eleven amino acids studied, none of the three antibiotics in any of the concentrations tested permitted sufficient growth to increase the titratable acidity beyond that of the blank, which was the medium that lacked both the essential

*In higher concentrations (400 μg per milliliter or more), both chlortetracycline and oxytetracycline retained sufficient activity after autoclaving (at pH 7.4 for 15 min. at 15 lb. pressure) to inhibit *S. faecalis* and the strains of *L. casei* in the complete synthetic medium (AAM).

TABLE III. REPLACEMENT OF AMINO ACIDS BY ANTIBIOTICS IN A SYNTHETIC AMINO ACID MEDIUM[2, 3]

AMINO ACID	CONCENTRATION IN CONTROL (μg/ML.)	TEST ORGANISM	TITRATABLE ACIDITY (ML. OF 0.05N NaOH) AFTER INCUBATION FOR 60 HOURS											
			CONTROL*	BLANK†	CHLORTETRACYCLINE‡			OXYTETRACYCLINE‡			CHLORAMPHENICOL‡		
					100	10	1	100	10	1	100	10	1
Arginine	10	*S. faecalis* 9790	7.8	1.2	1.0	1.1	1.1	1.1	1.1	1.0	0.6	0.6	1.2
Histidine	5	*S. faecalis* 9790	7.5	0.8	1.0	0.9	1.1	1.2	0.8	0.8	0.5	0.5	0.8
Isoleucine	10	*S. faecalis* 9790	8.2	1.5	0.8	1.3	1.4	0.5	1.6	1.6	0.5	0.4	1.6
Leucine	20	*S. faecalis* 9790	8.0	0.6	0.8	0.5	0.5	0.6	0.5	0.5	0.5	0.5	0.5
Lysine	30	*S. faecalis* 9790	7.6	0.8	0.8	0.8	0.8	1.2	0.8	0.8	0.5	0.5	0.7
Methionine	20	*S. faecalis* 9790	8.1	1.1	1.0	1.0	1.0	1.5	1.2	1.1	0.9	1.1	1.0
Threonine	20	*S. faecalis* 9790	7.4	0.4	0.5	0.5	0.5	0.5	0.4	0.4	0.5	0.5	0.5
Tryptophane	4	*S. faecalis* 9790	7.9	0.7	0.7	0.5	0.6	0.8	0.6	0.5	0.5	0.5	0.5
Valine	20	*S. faecalis* 9790	8.1	0.6	0.5	0.6	0.7	1.0	0.6	1.0	0.5	0.5	0.5
Phenylalanine	10	*L. casei* 9595	5.4	0.3	0.3	0.3	0.3	0.4	0.3	0.5	0.3	0.3	0.4
Tyrosine	10	*L. delbrueckii* LD5	5.2	0.8	1.2	0.9	0.9	0.9	0.8	1.0	0.4	0.6	0.7

*Amino acid present in the indicated concentration; no antibiotic.
†Indicated amino acid absent; no antibiotic.
‡Concentration in μg per milliliter added instead of the indicated amino acid before autoclaving for 15 minutes at 15 pounds pressure; organisms then added.

TABLE IV. REPLACEMENT OF VITAMINS BY CHLORTETRACYCLINE IN MICROBIOLOGIC ASSAYS

TEST ORGANISM	VITAMIN	CONCENTRATION OF VITAMINS IN CONTROL	TITRATABLE ACIDITY, ML. OF 0.1N NaOH (% LIGHT TRANSMISSION IN PYRIDOXINE ASSAY)				
			CONTROL*	CHLORTETRACYCLINE (μG/ML.) (NO VITAMIN)			
				0	1	10	100
L. casei, 7469	Folic acid	0.2 mμg/ml.	11.2	1.0	1.1	1.4	3.1
L. arabinosus	Niacin	0.1 μg/ml.	8.6	1.0	1.1	1.3	1.2
L. casei, 7469	Riboflavin	0.1 μg/ml.	6.7	1.2	1.1	1.1	1.4
L. citrovorum	Citrovorum factor	0.3 mμg/ml.	8.6	1.4			1.7
S. carlsbergensis	Pyridoxine	3.0 mμg/ml.	35.5	77.0		77.5	73.0

*Growth in the presence of the indicated (optimum) amount of vitamin and without chlortetracycline.

amino acid and the antibiotic. Under the conditions of this experiment, therefore, none of these antibiotics substituted for any of the amino acids in supporting the growth of these assay organisms.

An additional experiment was carried out to determine whether unautoclaved antibiotics used in subinhibitory concentrations would replace any of the amino acids used in support of the growth of the microbiologic assay organisms. The experiment was essentially a repetition of that shown in Table III with the following changes: (1) the media were autoclaved *before* the antibiotics (unautoclaved) and the organisms were added; (2) the antibiotics were used in twofold dilutions ranging from 3.1 to 0.4 μg per milliliter for chlortetracycline, 1.6 to 0.2 μg per milliliter for oxytetracycline, and 6.3 to 0.8 μg per milliliter for chloramphenicol. The results were the same as those obtained with the concentrations of autoclaved antibiotics used in the previous experiment; none of the tubes containing antibiotic showed any increase in titratable acidity over that of the culture in the medium in which any one of the individual amino acids had been omitted.

Replacement of Vitamins by Aureomycin.—Studies were made to determine whether chlortetracycline added to media used in the microbiologic assay methods would replace the requirement for folic acid,* nicotinic acid, riboflavin, citrovorum factor, and pyridoxine for the growth of the specific organisms used in their assays. Chlortetracycline was added to the media in concentrations of 1, 10, and 100 μg per milliliter and mixtures were autoclaved before inoculating the test organisms, thus destroying the antibiotic activity of the chlortetracycline and simulating the conditions of routine assays of specimens, such as urines, for these vitamins.

The data, shown in Table IV, indicate that under the conditions stated, chlortetracycline did not replace nicotinic acid for the growth of *L. arabinosus,* riboflavin for the growth of *L. casei,* citrovorum factor for the growth of *L. citrovorum,* nor pyridoxine for the growth of *S. carlsbergensis.* It was repeatedly noted in this and other similar experiments, however, that when chlortetracycline in concentrations of 100 μg per milliliter was added to a vitamin-free casein hydrolysate medium and autoclaved, it supported one-fourth to

*Synthetic pteroylglutamic acid, obtained from Lederle Laboratories, was used in all of the tests referred to here as those for folic acid.

one-third maximal growth of *L. casei* in the absence of added folic acid. This aspect was investigated further and some of the findings will be presented later.

Chlortetracycline and Riboflavin.—In the course of the metabolic studies in persons receiving oral chlortetracycline,[1] special interest centered about the increased urinary excretion of riboflavin. The effect of chlortetracycline on the assay of riboflavin was, therefore, studied in somewhat greater detail. The growth response of *L. casei* (ATCC 7469) to riboflavin was measured over a range of concentrations from 0 to 0.1 μg per milliliter of the basal medium without chlortetracycline and also in the presence of 25, 50, and 100 μg per milliliter of the antibiotic; each of these concentrations of antibiotic were tested both autoclaved and unautoclaved prior to inoculation of the test organism.

TABLE V. EFFECT OF HIGH CONCENTRATIONS OF CERTAIN VITAMINS ON THE ACTIVITY OF CHLORTETRACYCLINE AGAINST SPECIFIC ASSAY ORGANISMS

VITAMIN AND (ASSAY ORGANISM)	CONCENTRATION OF VITAMIN (μG/ML.)	TITRATABLE ACIDITY, MILLILITERS OF 0.1N NaOH — CHLORTETRACYCLINE, MICROGRAMS PER MILLILITER (NOT AUTOCLAVED)									
		0	0.4	0.8	1.6	3.1	6.3	12.5	25	50	100
Riboflavin (*L. casei*, 7469)	0.0	1.1	1.3	1.3	1.1	1.1	1.0	1.0	0.9	0.9	0.9
	0.25	8.2	8.0	7.5	5.7	3.8	1.9	1.4	1.3	1.3	1.3
	0.5	8.0	7.8	7.2	6.0	3.1	1.5	1.1	1.0	0.9	0.9
	2.5	8.5	8.6	7.8	5.0	1.6	1.2	1.1	0.9	1.0	1.0
	5.0	8.5	8.2	7.2	5.3	2.4	1.3	1.1	1.0	1.0	1.0
	10.0	8.2	8.6	7.4	4.2	2.6	1.1	1.1	1.0	1.1	1.1
Folic acid (*L. casei*, 7469)	0.0	0.7	0.9	1.0	1.4	1.0	0.7	0.8	0.8	0.8	0.9
	0.001	9.2	7.2	6.7	3.4	1.3	1.4	0.9	0.9	1.0	0.7
	0.01	9.6	8.4	7.1	4.9	1.8	0.9	1.1	0.9	1.1	1.0
	1.0	9.8	8.9	5.9	4.4	1.6	1.0	0.9	0.9	0.8	0.8
	10.0	9.7	9.0	6.7	3.8	1.1	1.0	1.3	0.9	1.1	1.4
Citrovorum factor (*L. citrovorum*)	0.0	1.3	1.6	1.6	1.5	1.4	1.5	1.4	1.7	1.6	1.5
	0.05	8.4	9.3	9.6	9.4	8.7	9.1	8.8	7.5	4.2	2.5
	0.5	9.7	10.4	11.0	10.8	10.8	9.8	8.8	8.1	5.7	1.7
	1.0	10.7	11.8	11.1	10.8	10.4	10.3	8.5	7.6	4.9	2.0
	10.0	10.1	10.3	10.8	11.1	11.2	9.8	10.7	8.1	4.9	2.5
	50.0	9.7	11.2	11.1	10.9	10.9	10.6	9.5	6.7	3.4	1.6

The results are depicted in Fig. 1. The data show clearly that the autoclaved chlortetracycline, in the concentrations used, had no effect on the growth response of *L. casei* to the graded amounts of riboflavin. Unautoclaved chlortetracycline in each of the concentrations tested completely inhibited the growth of the organisms at all of the concentrations of riboflavin that were used. The activity of chlortetracycline, therefore, was not counteracted by riboflavin under the conditions of this experiment.

Effect of Riboflavin, Folic Acid, and Citrovorum Factor on Sensitivity of Assay Organisms to Chlortetracycline.—The effect of large concentrations of certain nutrients required by some of the microbiologic test organisms upon the inhibitory action of chlortetracycline against these organisms was investigated. In those experiments, therefore, the media, including the vitamins, were autoclaved before the antibiotic was added. The results of some of these studies

are shown in Table V. These data indicate that the sensitivity of *L. casei* to chlortetracycline was not affected by the addition of riboflavin in concentrations ranging from 0.25 to 10.0 μg per milliliter, nor by the addition of folic acid in concentrations ranging from 0.001 to 10.0 μg per milliliter of medium. Likewise, the susceptibility of *L. citrovorum,* which is much more resistant to this antibiotic, was not altered by the addition of citrovorum factor (leucovorin) in concentrations ranging from 0.05 to 50 μg per milliliter.

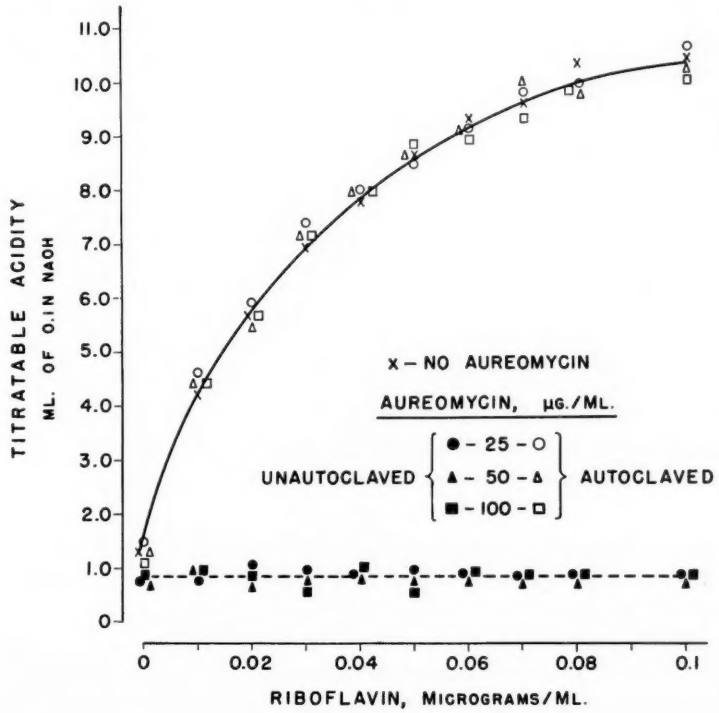

Fig. 1.—The growth response of *L. casei* (ATCC 7469) to riboflavin as measured by titratable acidity, with bromthymol blue as indicator, after incubation for 72 hours at 37° C. is shown in the solid curve. The values for the cultures containing riboflavin to which chlortetracycline was added before autoclaving all fell on or close to this curve, indicating lack of effect of the indicated amounts of autoclaved chlortetracycline. In the presence of the same concentrations of unautoclaved chlortetracycline, growth of the organism was completely inhibited at all levels of riboflavin, all values falling along the broken horizontal line, indicating that the riboflavin did not counteract the antibiotic activity of the chlortetracycline.

Folic Acid Activity of Chlortetracycline.—The finding of folic acid activity in samples of chlortetracycline after autoclaving suggests two possibilities. Either the degradation of chlortetracycline during autoclaving in the alkaline medium resulted in the formation of a compound with folic acid activity for *L. casei,* or small amounts of folic acid were contained in the preparations of chlortetracycline as a contaminant. That this material was folic acid and not citrovorum factor was demonstrated by the failure of autoclaved chlortetracycline to support the growth of *L. citrovorum* and by the observation that the folic acid activity in the antibiotic was inhibited by Aminopterin; the evidence is presented in Table VI.

TABLE VI. EVIDENCE FOR FOLIC ACID ACTIVITY OF CHLORTETRACYCLINE*

EVIDENCE FOR:	INGREDIENTS ADDED TO BASAL MEDIUM				TITRATABLE ACIDITY (ML. OF 0.1N NaOH)
	FOLIC ACID (MμG/ML.)	AUTOCLAVED CHLORTETRACYCLINE (μG/ML.)	CITROVORUM FACTOR (MμG/ML.)	AMINOPTERIN (MμG/ML.)	
A. Folic acid activity of autoclaved chlortetracycline on *L. casei*, 7469	0	0			0.8
	0.02	0			1.9
	0.04	0			3.0
	0.06	0			4.1
	0	50			2.0
	0	100			2.5
	0	200			3.2
B. Failure of autoclaved chlortetracycline to support the growth of *L. citrovorum*		0	0		1.4
		0	0.09		4.0
		0	0.18		6.5
		0	0.27		8.6
		50	0		1.5
		100	0		1.7
		200	0		1.5
C. Reversal by Aminopterin of the folic acid activity found in autoclaved chlortetracycline	0	200		0	2.9
	0	200		0.02	1.7
	0	200		0.04	1.2
	0.04	0		0.02	1.1
	0.04	0		0.04	0.9

*Lot No. 4423-105 was used in this experiment.

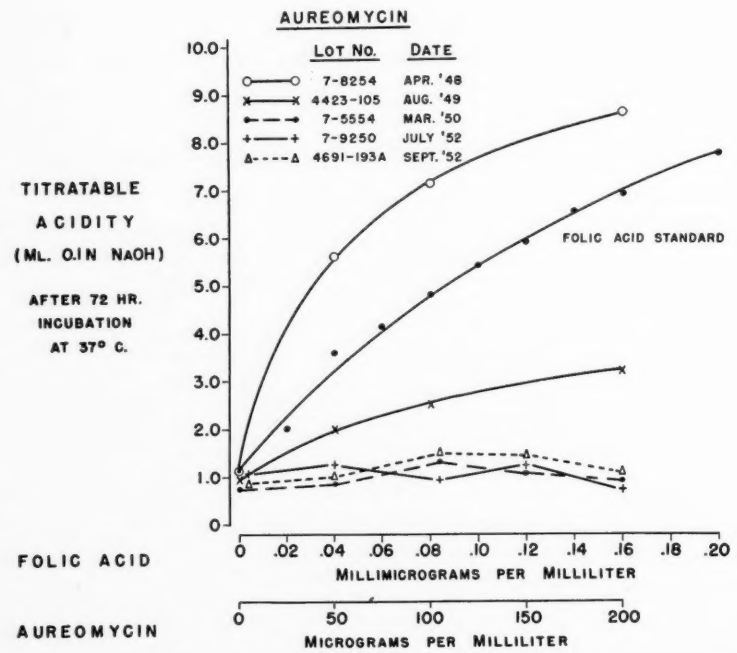

Fig. 2.—Standard growth response curve of *L. casei* to graded concentrations of folic acid and effect of substituting five different lots of autoclaved chlortetracycline for the folic acid. Considerable amounts of folic acid activity were supplied by the two early lots but the three that were received on or after March, 1950, showed little or no such activity in the concentrations used. Higher concentrations (400 μg per milliliter or more) retained sufficient antibiotic activity after autoclaving to inhibit the growth of the test organism.

Subsequent studies have demonstrated that the folic acid activity of autoclaved chlortetracycline was greatest in the early lots of this antibiotic, and that more recent lots contained none or only negligible amounts. The results of assays of five lots of chlortetracycline for folic acid activity against *L. casei* are shown in Fig. 2.* The findings suggest that folic acid was present in small amounts as a contaminant in early preparations of Aureomycin and that subsequent refinements in manufacture (at least since March, 1950) have eliminated this contaminant.†

DISCUSSION

The results of assays of antibiotics and of tests for sensitivity of microorganisms to antibiotics may be considerably influenced by the conditions of the tests—for example, by certain constituents of the medium, its pH, the size of the bacterial inoculum, the rate and stage of growth, etc.—and the effects of these factors vary with the different antibiotics.[12, 13] In addition, certain antibiotics may have striking effects in enhancing the nutrition of animals under appropriate conditions,[14, 15] and the same may be true for the growth of certain microorganisms. In the course of investigations on the nutritional effects of antibiotics, particularly chlortetracycline, in man, microbiologic assay methods were used to measure some of the essential nutrients in blood, urine, and feces. It became necessary, therefore, to determine whether the presence of antibiotics in the materials to be assayed had any inhibitory or enhancing effects under the conditions of the special assays used. The results of some of the studies carried out for that purpose have been presented here.

In the tests for sensitivity of the microbiologic assay organisms to various antibiotics, the results obtained with the use of the appropriate complete microbiologic assay media were, in general, comparable to those obtained in the more complex bacteriologic media employed routinely for sensitivity tests. The instances in which there were gross discrepancies warrant further study to determine whether these were real and if so, which factors were responsible. Such information may shed light on the mechanisms of the antimicrobial action of the antibiotics on the one hand, and their possible role in the nutrition of microorganisms on the other. Differences in the results of sensitivity tests done in different bacteriologic media may depend on similar factors. Only selected aspects bearing directly on the problems concerned in the microbiologic assays of certain nutritional elements, namely, those used in the study on the effect of chlortetracycline, were presented in this study.

In the conduct of the microbiologic assays, the media are usually autoclaved before inoculating the test organisms. The effect of this procedure on

*In these and other experiments it was found that in media containing concentrations of 400 μg per milliliter or more of chlortetracycline, sufficient antibiotic activity was retained after autoclaving to inhibit the strain of *L. casei*.

†The presence of folic acid activity in one of the early lots of chlortetracycline (Lot 4423-105) was confirmed by Dr. Harry P. Broquist of Lederle Laboratories. "Two hundred milligrams of this preparation were autoclaved for 30 minutes in 20 ml. of 0.05N NaOH, cooled, and the folic acid activity adsorbed on charcoal at pH 2. The charcoal was then treated with alcohol and ammonia to elute the folic acid, and this eluate concentrated to 0.5 ml. and chromatogrammed on paper in aqueous 1 per cent K_2HPO_4. The presence of folic acid was demonstrated by bioautographic technique with *S. faecalis*." (Personal communication, Nov. 14, 1952.)

the activity of the antibiotics varies with the antibiotic, its concentration, the pH of the medium in which the procedure is carried out, etc.; this must, therefore, be determined for each antibiotic under the particular conditions of the assay method that is used. It was shown here that in the media used, at pH 7.4, and in concentrations of 100 μg per milliliter or less, all of the inhibitory activity of chlortetracycline and oxytetracycline against the assay organisms tested was destroyed, but that of chloramphenicol remained intact. In higher concentrations of chlortetracycline of oxytetracycline (400 μg or more per milliliter) enough activity remains to inhibit the more sensitive assay organisms, and when the concentration is sufficiently high, the residual activity after autoclaving may be sufficient to inhibit the more resistant strains, such as *L. citrovorum* and *L. arabinosus;* such high concentrations may conceivably be present in the feces of patients under treatment.

In the present study it was shown that chlortetracycline, oxytetracycline, and chloramphenicol could not replace any of eleven amino acids necessary to support the growth of the assay organisms. It was also shown that chlortetracycline, in amounts of 1, 10, or 100 μg per milliliter, failed to supply the growth requirements of certain assay organisms for niacin, riboflavin, citrovorum factor, or pyridoxine. In a concentration of 100 μg per milliliter, however, some of the lots of chlortetracycline used did supply sufficient folic acid activity to support about one-fourth to one-third maximal growth of *L. casei.* When this was investigated further, it was shown that the folic acid activity was present in significant amounts only in the earlier lots of chlortetracycline, presumably as a contaminant. This contaminant has apparently been removed in the subsequent refinements of manufacture so that folic acid activity can no longer be demonstrated in recent lots of this antibiotic.

Whether or not the presence of folic acid in the Aureomycin was a factor in the hematopoietic response attributed to this antibiotic in patients with pernicious anemia or of nutritional macrocytic anemia, as reported by Lichtman, Ginsberg, and Watson[16] cannot be determined without more data than are at present available. Moreover, while it seems unlikely that the possible presence of folic acid in the lot of antibiotic used by Waisman and associates[17] could explain their observation that Aureomycin counteracted the toxic effects of large amounts of aminopterin in the diet of rats, this possibility cannot be entirely ignored, particularly in view of the recent demonstration by Zakrzewski and Nichol[18] that a sample of Aminopterin contained up to 24 per cent pteroylglutamic acid.

Foster and Pittillo,[19] using small inocula of *E. coli,* strain B, on the surface of glucose-salt agar plates containing mixtures of antibiotics and pure metabolites, were able to demonstrate that many of the metabolites they tested could reverse the inhibition of growth of this strain caused by certain levels of various antibiotics. In their experiments, the action of Aureomycin on this strain of *E. coli* was reversed by riboflavin, and quantitative studies showed this reversal to be competitive. Aureomycin was also found to inhibit competitively

the utilization of riboflavin by a riboflavinless mutant of *B. subtilis*. In the experiments presented in this paper, large doses of riboflavin and also of folic acid failed to reverse the inhibition of growth of *L. casei* by chlortetracycline and high concentrations of citrovorum factor failed to alter the inhibition of *L. citrovorum* by this antibiotic in the media used.

SUMMARY

Tests for sensitivity of certain assay organisms to various antibiotics, when carried out in the defined media used for the assay of amino acids and vitamins, usually gave results which were similar to those obtained in the routine broth or agar media. There were some significant discrepancies which warrant further study.

Chlortetracycline, oxytetracycline, and chloramphenicol, when added in concentrations of 1, 10, and 100 μg per milliliter and autoclaved failed to replace any of eleven amino acids essential for the growth of appropriate organisms in a synthetic amino acid medium. Likewise, none of these three antibiotics, when added in subinhibitory concentrations after the synthetic medium was autoclaved, could replace any of the eleven amino acids in supporting the growth of the appropriate organisms.

The antibiotic activity of chlortetracycline and oxytetracycline, in concentrations of 100 μg per milliliter or less in a synthetic amino acid medium at pH 7.4 was completely destroyed by autoclaving; the activity of chloramphenicol was unaffected under the same conditions.

Chlortetracycline added to a basal medium in concentrations of 25, 50, and 100 μg per milliliter and autoclaved had no effect on the growth response of *L. casei* to graded suboptimal amounts of riboflavin, and the inhibitory activity against this organism of unautoclaved chlortetracycline in the same concentrations was not counteracted by riboflavin in amounts up to 0.1 μg per milliliter.

Chlortetracycline, in concentrations of 1, 10, or 100 μg per milliliter, after autoclaving did not replace optimum amounts of niacin, riboflavin, citrovorum factor, nor pyridoxine in supporting the growth of *L. arabinosus, L. casei* 7469, *L. citrovorum,* and *S. carlsbergenesis,* respectively, in their appropriate media, nor could unautoclaved chlortetracycline in subinhibitory concentrations replace riboflavin. Under similar circumstances, however, autoclaved chlortetracycline at a concentration of 100 μg per milliliter was found to support the growth of *S. faecalis* to an extent equivalent to one-fourth or one-third of that supplied by the optimum amount of folic acid.

The folic acid activity of chlortetracycline was found to be a contaminant of early lots and could not be demonstrated in recent lots of this antibiotic.

The inhibition of *L. casei* 7469 by unautoclaved chlortetracycline was not reversed by large doses of riboflavin or of folic acid and the inhibition of *L. citrovorum* by this antibiotic could not be counteracted by high concentrations of citrovorum factor.

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IN VITRO SUSCEPTIBILITY OF RECENTLY ISOLATED STRAINS OF PROTEUS TO TEN ANTIBIOTICS

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ORGANISMS of the genus Proteus have gained prominence in recent years because of an apparent increase in the frequency with which they are being encountered in various chronic suppurative lesions, particularly following antibiotic therapy. The significance of Proteus infections, however, may be out of proportion to their frequency because of their relative resistance to treatment with most of the antibiotics that are in common use.[1-5]

A study of the susceptibility of strains of Proteus to various antibiotics was previously reported from this laboratory[6]; that study dealt with strains isolated in the routine bacteriologic laboratory of this hospital in 1949 and tested with seven antibiotics then available. The present study was undertaken to determine whether the antibiotic susceptibility of currently isolated organisms in this group has changed, in a manner similar to that noted with the staphylococcus,[7] in the face of the widespread use of antibiotics. It was also of interest to determine: (1) the relative activity of some of the more important antibiotics that have become available since the earlier study was completed; (2) whether the degree of resistance of the strains to certain antibiotics could be correlated with previous antibiotic treatment of the patients from whom they were isolated; and (3) whether antibiotic susceptibility could be correlated with certain biochemical reactions used to characterize the different species of Proteus.

MATERIALS AND METHODS

The 119 strains of Proteus included in this study were isolated from patients under treatment at the Boston City Hospital between December, 1953, and April, 1954. The first 110 of these strains were collected in a random manner from the general bacteriologic laboratory* without reference to source, prior treatment, or consideration of their pathogenic role. The last nine strains, however, were nonspreading organisms isolated in the same manner at the end of the study in order to add to the relatively small number of such strains included up to that time and thus permit a somewhat better comparison with the larger number of "swarmers." Two or three strains were obtained from each of nine patients, either at different times or from different sources at the same time. Reasonable assurance of purity was obtained either by selection, when possible, of a single colony from a streaked agar plate during the first hours of growth, or by the selection of a typical isolated colony from the surface of an SS agar plate on which swarming is inhibited. An occasional contaminant was detected by atypical fermentations or other biochemical reactions during the study; such strains were "repurified" and then restudied.

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Aided by a grant from the United States Public Health Service.
Received for publication June 7, 1954.
*We are indebted to Miss Marion E. Lamb and Miss A. Kathleen Daly for isolating all of the strains.

The strains selected were classified as Proteus on the basis of their ability to split urea within forty-eight hours by the method of Stuart and associates.[8] In addition to urease production and swarming, certain biologic reactions were selected from among those suggested by Rustigian and Stuart[9] in order to divide the strains into the four species essentially as defined by these authors.* The classification used is shown in Table I. Some of the fermentation reactions were quite slow as, for example, that of sucrose by strains of *P. mirabilis* and *P. rettgeri,* some of which required up to three weeks of incubation. All cultures were streaked singly on heart infusion agar plates in order to observe the type and degree of swarming during incubation at 37° C. Other procedures were also carried out but were not used in the classification and are, therefore, not considered.

TABLE I. CRITERIA FOR DIVISION OF STRAINS OF PROTEUS INTO SPECIES*

REAGENT	P. VULGARIS	P. MIRABILIS	P. MORGANI	P. RETTGERI
Sucrose	AG, rapid	A, slow	--	A, slow
Maltose	AG, rapid	--	--	--
Mannitol	--	--	--	A, rapid
Indole	+	--	+	+
Citrate	-- or +	+	--	+
Urea	+	+	+	+

A = Acid formed.
AG = Acid and gas formed.
+ = Positive reaction.
-- = No fermentation or negative reaction.
Rapid = Less than 48 hours.
Slow = Three to 21 days.
*After Rustigian and Stuart.[9]

The fermentations were carried out with 0.5 per cent solutions of the sugars in phenol red broth (Difco). The test for indole was carried out with Kovac's reagent after growth for twenty-four hours in 1 per cent Bacto-tryptose (Difco). The test employing Kovac's reagent was found to be superior to the Ehrlich-Boehme and Gnezda tests for indole.† Utilization of citrate was observed in Bacto-Simmons Citrate agar with readings made at twenty-four hours.

The tests for antibiotic susceptibility‡ were carried out by the twofold agar-plate dilution method as in previous studies from this laboratory.[10, 11] As the organisms generally did not swarm at concentrations of antibiotic near those which were inhibitory, there was usually no difficulty in reading the tests. Where swarming made end points indistinct, however, the tests were repeated using a single strain to a plate. The susceptibility of a strain to an antibiotic is here defined as the minimum concentration of the antibiotic in the agar on which there is no visible growth after incubation at 37° C. for twenty-four hours. All values are expressed in micrograms of antibiotic per milliliter of medium.§ No arbitrary definitions of "susceptible" and "resistant" were used in the present study, these terms being employed only in a relative sense within the range of concentrations used in the tests.

The following antibiotics were used: Crystalline potassium penicillin G (1 unit considered equal to 0.6 μg), and streptomycin sulfate, both obtained commercially; chlortetracycline (Aureomycin) hydrochloride and tetracycline (Achromycin) hydrochloride supplied by Dr. Stanton M. Hardy of Lederle Laboratories Division, American Cyanamid Co.; oxytetracycline (Terramycin) hydrochloride furnished by Dr. Gladys L. Hobby of Chas. Pfizer Co.; neomycin sulfate obtained from Dr. H. F. Hailman of the Upjohn Co.; chloramphenicol (Chloromycetin) supplied by Dr. E. A. Sharp of Parke, Davis & Company; erythromycin

*See also Bergey's Manual of Determinative Bacteriology, ed. 6, Williams & Wilkins Co., 1948, p. 486.

†Details of these tests for indole are given in Difco Manual, ed. 9, 1953, p. 53.

‡Carried out with the assistance of Clare Wilcox and Ann Najarian.

§The designation ">400" in the tables and figures denotes growth in 400 μg per milliliter, the highest concentration used; it was not determined whether greater concentrations were inhibitory.

(Ilotycin) furnished by Dr. J. W. Smith of Eli Lilly & Co.; bacitracin (51.7 units per mg.) obtained from Dr. L. W. Smith of Commercial Solvents Corp. and polymyxin B sulfate (Aerosporin) supplied by Dr. Donald F. Searle of Burroughs Wellcome & Co.

RESULTS

Classification of Strains Into Species, Relation to Sources and to Swarming.—The sources of the strains included in this study and their classification into species according to the criteria enumerated previously are listed in Table II. Almost three-fourths of the strains were classified as *P. mirabilis,* and there were only 12 strains of *P. vulgaris,* 15 of *P. morgani,* and 6 of *P. rettgeri.* Because of the small numbers of strains other than *P. mirabilis,* no significance can be attached to the relative frequency with which strains of the different species were isolated from various sources. Swarming on ordinary nutrient agar at 37° C. was exhibited by 84 of the 86 strains of *P. mirabilis* but by only 2 strains each of *P. vulgaris* and *P. morgani,* and by none of those of *P. rettgeri.*

TABLE II. SOURCES FROM WHICH STRAINS OF PROTEUS WERE CULTURED

SOURCE	NUMBER	SPECIES OF PROTEUS			
		P. VULGARIS	P. MIRABILIS	P. MORGANI	P. RETTGERI
Urine	57	11	32	8	6
Superficial infections*	16	0	14	2	0
Sputum (7) and throat (6)	13	1	11	1	0
Feces	11	0	8	3	0
Draining sinuses†	10	0	10	0	0
Otitis	7	0	7	0	0
Peritonitis	3	0	2	1	0
Bile[1] and spinal fluid[1]	2	0	2	0	0
All sources	119	12	86	15	6

*Gangrene, 5; infected wounds, 3; decubitus ulcers, 3; diabetic ulcers, 2; burns, 1; abscess of buttock, 1; and abscess of mouth, 1.
†Osteomyelitis, 8; abdominal, 2.

Antibiotic Susceptibility of All Strains.—The susceptibility of all 119 strains of Proteus to each of the antibiotics is depicted graphically in Fig. 1 in a manner similar to that used in previous studies with these and other organisms.[6, 10] In this figure it is seen that the susceptibility of different strains to some antibiotics, namely neomycin, erythromycin, bacitracin, and polymyxin, is more or less uniform, while their susceptibility to other antibiotics varies widely, a significant proportion of the strains being quite susceptible, while most of the rest are highly resistant to each of the agents. The curves representing the cumulative per cent of strains with progressively increasing resistance to the latter antibiotics are, therefore, irregular in shape. The only striking comparison which is evident from this figure, is the somewhat greater susceptibility of many of the strains to tetracycline as compared with their susceptibility to its two analogues.

Relation of Antibiotic Susceptibility to the Species of Proteus.—Because the irregular shapes of the curves depicted in Fig. 1 could be the resultant of differences in susceptibility related to the species of the strains, the results were analyzed from that point of view. The susceptibility of each of the strains of all species other than *P. mirabilis* are given in Table III. From this table it is seen that the susceptibility of the strains of all three species was similar for all anti-

TABLE III. SUSCEPTIBILITY OF THIRTY-THREE STRAINS OF PROTEUS (OF SPECIES OTHER THAN P. MIRABILIS) TO 10 ANTIBIOTICS

STRAIN	SOURCE	PREVIOUS ANTIBIOTIC THERAPY†	SPECIES	MINIMUM INHIBITING CONCENTRATION, MICROGRAMS PER MILLILITER*							
				CHLOR-TETRA-CYCLINE	OXYTETRA-CYCLINE	TETRA-CYCLINE	CHLORAM-PHENICOL	STREPTO-MYCIN	NEOMYCIN	PENI-CILLIN	ERYTHRO-MYCIN
14	Urine	T	*P. vulgaris*	12.5	12.5	25	25	50	50	>400	400
15	Urine	P	*P. vulgaris*	12.5	6.3	12.5	200	>400	50	>400	400
40	Urine	P	*P. vulgaris*	6.3	25	12.5	200	>400	25	>400	>400
43	Urine	P, Ct	*P. vulgaris*	6.3	12.5	6.3	200	>400	50	>400	>400
47	Urine	P, S	*P. vulgaris*	3.1	3.1	6.3	12.5	50	50	>400	400
58	Urine	None	*P. vulgaris*	6.3	3.1	12.5	200	>400	25	>400	>400
71	Urine	P	*P. vulgaris*	6.3	12.5	6.3	200	>400	25	>400	>400
79	Urine	P	*P. vulgaris*	12.5	6.3	3.1	200	50	50	>400	400
83	Urine	None	*P. vulgaris*	3.1	3.1	3.1	200	>400	50	>400	400
84	Urine	P	*P. vulgaris*	3.1	3.1	3.1	100	>400	50	>400	400
95	Sputum	None	*P. vulgaris*	12.5	12.5	6.3	25	50	25	>400	200
114	Urine	P	*P. vulgaris*	12.5	6.3	12.5	100	>400	50	>400	>400
35	Urine	None	*P. morgani*	6.3	3.1	6.3	100	50	25	>400	>400
39	Urine	P, Ct	*P. morgani*	>400	>400	>400	200	>400	12.5	>400	>400
46	Feces	Ot	*P. morgani*	3.1	3.1	6.3	12.5	50	25	>400	>400
48	Urine	T	*P. morgani*	>400	>400	>400	>400	>400	25	>400	400
57	Urine	P, T, Cm	*P. morgani*	>400	>400	>400	>400	>400	25	>400	>400
59	Urine	None	*P. morgani*	12.5	3.1	12.5	12.5	>400	25	>400	>400
69	Throat	None	*P. morgani*	6.3	6.3	6.3	25	25	12.5	>400	400
99	Urine	P	*P. morgani*	>400	>400	>400	>400	>400	12.5	>400	>400
106	Peritoneum	P	*P. morgani*	12.5	12.5	12.5	50	12.5	25	>400	>400
107	Abscess	P	*P. morgani*	12.5	12.5	12.5	25	12.5	25	>400	400
109	Urine	Ot, T, Cm	*P. morgani*	50	12.5	12.5	200	>400	50	>400	>400
111	Abscess	P	*P. morgani*	100	25	12.5	50	12.5	25	200	>400
116	Feces	P	*P. morgani*	25	25	12.5	12.5	12.5	25	>400	>400
118	Urine	P	*P. morgani*	25	12.5	12.5	25	>400	50	>400	>400
120	Feces	B, Pm	*P. morgani*	>400	>400	200	100	>400	100	>400	50
74	Urine	P	*P. rettgeri*	>400	>400	>400	>400	>400	400	>400	50
100	Urine	P, S	*P. rettgeri*	>400	>400	>400	400	>400	50	>400	400
108	Urine	P, S	*P. rettgeri*	>400	>400	>400	>400	>400	25	>400	400
113	Urine	P, S	*P. rettgeri*	12.5	6.3	6.3	100	>400	25	>400	400
115	Urine	P	*P. rettgeri*	12.5	6.3	12.5	100	>400	50	>400	>400
121	Urine	P, S, Ct	*P. rettgeri*	>400	>400	400	12.5	>400	25	>400	>400

*Readings after incubation at 37° C. for 24 hours. All strains grew freely in 400 μg per milliliter of bacitracin and polymyxin B.

†T = Tetracycline; P = Penicillin; Ct = Chlortetracycline; Ot = Oxytetracycline; Cm = Chloramphenicol; S = Streptomycin; B = Bacitracin; Pm = Polymyxin.

biotics except those of the tetracycline group; within this group, all strains of *P. vulgaris* were susceptible to about the same degree to all three tetracycline analogues, while those of *P. morgani* and *P. rettgeri* varied, some being as susceptible as those of *P. vulgaris* while others were resistant. To simplify further comparisons, it seemed justified, therefore, to group the strains of these three species together and to compare them with all the strains of *P. mirabilis.* The results are shown in Table IV and in Fig. 2.

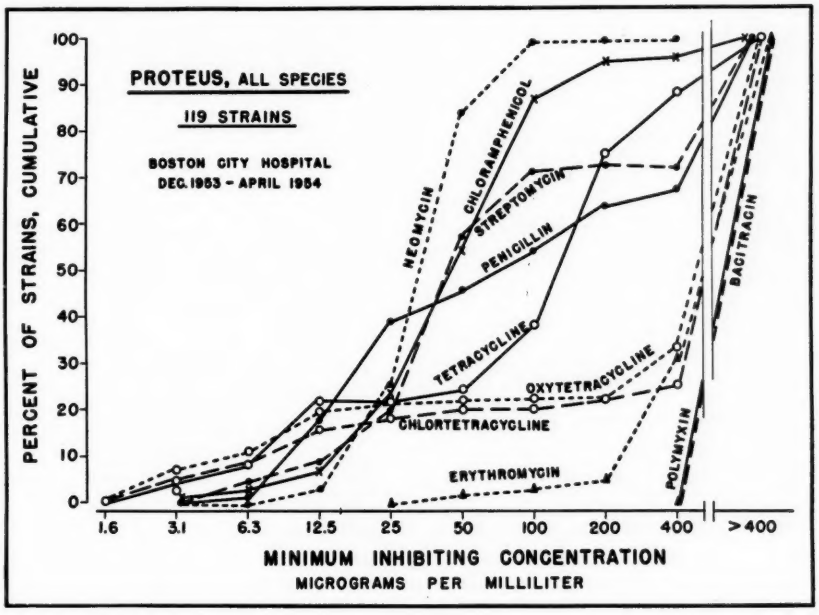

Fig. 1.

From Table IV it is seen that, with respect to neomycin, erythromycin, bacitracin, and polymyxin, the distribution of strains according to their susceptibility was the same for *P. mirabilis* and for the other species. However, there was a clear difference between strains of *P. mirabilis* and those of the three other species with respect to their susceptibility to the three tetracycline analogues. The strains of *P. mirabilis* were moderately or highly resistant to chlortetracycline and oxytetracycline and appreciably more susceptible to tetracycline, while those of *P. vulgaris, P. morgani,* and *P. rettgeri,* considered together, were predominantly susceptible with only a small proportion of them resistant to all three tetracycline analogues. From Table III it is apparent that the strains other than those of *P. mirabilis* that were resistant to the three tetracycline antibiotics represent only a part of those of *P. morgani* and *P. rettgeri,* while those of *P. vulgaris* were all susceptible to each of the three antibiotics.

It is also seen from Table IV that the strains of *P. mirabilis* were fairly uniform in their susceptibility to chloramphenicol, while those of the other species showed considerable variation in susceptibility to that agent. In this

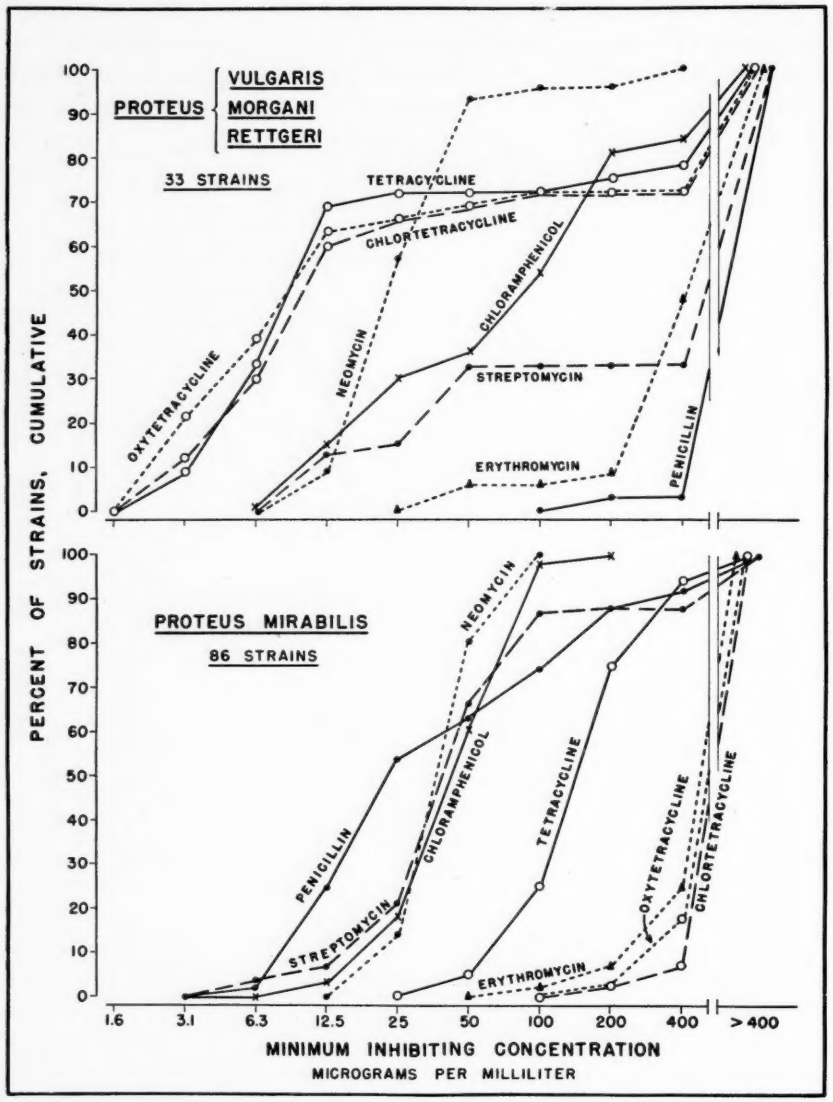

Fig. 2.

respect, the three species other than *P. mirabilis* did not show clear differences among each other, although, as seen in Table III, none of the strains of *P. vulgaris* were highly resistant. There was also a smaller proportion of strains of *P. mirabilis* resistant to streptomycin as compared with those of the other species and, as noted in Table III, all the strains of *P. rettgeri* were resistant to that antibiotic, whereas one-third and one-half of those of *P. vulgaris* and *P. morgani,* respectively, were moderately susceptible.

The most striking difference among the species was evident in their susceptibility to penicillin. The strains of *P. mirabilis* varied widely, most of them

being moderately susceptible; those of the other species were all highly resistant except for one strain that was moderately resistant to this antibiotic.

The difference between the susceptibility of strains of *P. mirabilis* and those of the other three species combined are also depicted graphically in Fig. 2; the curves for bacitracin and polymyxin are omitted from this figure since all strains were resistant to 400 μg. per milliliter of these two agents. This figure shows clearly the similarity of the effects of the three tetracycline antibiotics on the 33 strains of *P. vulgaris, P. morgani,* and *P. rettgeri;* the first ascending portions of these curves are constituted largely of strains of *P. vulgaris* and the terminal rise represents the resistant strains which include none of this species (cf. Table III). The lower panel of this figure depicts the greater inhibitory effect of tetracycline as compared with its two analogues against the strains of *P. mirabilis.*

TABLE IV. COMPARISON OF ANTIBIOTIC SUSCEPTIBILITIES OF EIGHTY-SIX STRAINS OF P. MIRABILIS WITH THOSE OF THIRTY-THREE STRAINS OF OTHER SPECIES OF PROTEUS

ANTIBIOTIC	SPECIES OF PROTEUS	NUMBER OF STRAINS INHIBITED BY:								
		3.1*	6.3	12.5	25	50	100	200	400	>400
Chlortetracycline	*P. mirabilis*	0	0	0	0	0	0	2	4	80
	Others†	4	6	10	2	1	1	0	0	9
Oxytetracycline	*P. mirabilis*	0	0	0	0	0	0	2	13	71
	Others	7	6	8	3	0	0	0	0	9
Tetracycline	*P. mirabilis*	0	0	0	0	4	17	43	15	7
	Others	3	8	12	1	0	0	1	1	7
Chloramphenicol	*P. mirabilis*	0	1	2	13	36	33	1	0	0
	Others	0	0	5	5	2	6	9	1	5
Streptomycin	*P. mirabilis*	0	3	3	13	38	17	1	0	11
	Others	0	0	4	1	6	0	0	0	22
Neomycin	*P. mirabilis*	0	0	0	12	57	17	0	0	0
	Others	0	0	3	16	12	1	0	1	0
Penicillin	*P. mirabilis*	0	1	20	25	8	10	11	4	7
	Others	0	0	0	0	0	0	1	0	32
Erythromycin	*P. mirabilis*	0	0	0	0	0	1	5	16	64
	Others	0	0	0	0	2	0	1	13	17
Bacitracin	*P. mirabilis*	0	0	0	0	0	0	0	0	86
	Others	0	0	0	0	0	0	0	0	33
Polymyxin	*P. mirabilis*	0	0	0	0	0	0	0	0	86
	Others	0	0	0	0	0	0	0	0	33

*Micrograms per milliliter.
†*P. vulgaris,* 12 strains; *P. morgani,* 15 strains; and *P. rettgeri,* 6 strains.

Also readily seen from a comparison of the upper and lower panels of Fig. 2 is the strikingly greater inhibitory action of penicillin against the strains of *P. mirabilis* as compared with the resistance of the other species. Streptomycin, and to a less extent chloramphenicol, are likewise seen to be more active against a larger proportion of strains of *P. mirabilis* as compared with their activity against the other species. The curves representing the activity of neomycin and erythromycin are similar in the two portions of this figure.

Relation to Swarming.—The swarming activity of the strains showed no clear relation to the susceptibility to any of the antibiotics. The two non-spreading strains of *P. mirabilis* were indistinguishable from the others in their susceptibility to each antibiotic. The two strains each of *P. vulgaris* and *P.*

TABLE V. ANTIBIOTIC SUSCEPTIBILITY OF MULTIPLE STRAINS OF PROTEUS OBTAINED FROM THE SAME PATIENTS AT THE SAME OR AT DIFFERENT TIMES

PATIENT	STRAIN	DATE	SOURCE	PREVIOUS ANTIBIOTIC THERAPY*	SPECIES	MINIMUM INHIBITING CONCENTRATION, MICROGRAMS PER MILLILITER						
						CHLORTETRACYCLINE	OXYTETRACYCLINE	TETRACYCLINE	CHLORAMPHENICOL	STREPTOMYCIN	NEOMYCIN	PENICILLIN
E. M.	22	Dec. 12	Ulcer	P	*P. mirabilis*	>400	400	200	50	25	50	>400
	28	Feb. 9	Ulcer	P, Pm	*P. mirabilis*	>400	400	100	50	50	50	>400
	54	Feb. 17	Ulcer	P, Pm, Cm	*P. mirabilis*	>400	>400	200	50	50	100	>400
E. H.	49	Feb. 15	Ulcer	P	*P. mirabilis*	>400	>400	100	50	50	50	200
	50	Feb. 15	Spinal fluid	P	*P. mirabilis*	>400	>400	100	50	50	50	25
	110	Mar. 21	Ulcer	P, T	*P. mirabilis*	>400	>400	200	100	50	50	200
H. G.	15	Jan. 28	Urine	P	*P. vulgaris*	12.5	6.3	12.5	200	>400	50	>400
	31	Feb. 1	Feces	P	*P. mirabilis*	>400	>400	400	100	100	100	200
	43	Mar. 12	Urine	P, T	*P. vulgaris*	6.3	12.5	6.3	200	>400	50	>400
B. G.	44	Feb. 12	Bile	T	*P. mirabilis*	>400	>400	200	100	50	100	25
	72	Feb. 25	Peritoneum	T	*P. mirabilis*	>400	>400	200	100	50	50	25
K. B.	51	Feb. 17	Throat	P, Ct	*P. mirabilis*	>400	>400	100	200	100	50	12.5
	61	Feb. 23	Urine	P, Ct, S	*P. mirabilis*	>400	>400	200	100	100	50	12.5
M. B.	63	Feb. 23	Urine	P	*P. mirabilis*	>400	>400	200	50	100	50	12.5
	66	Feb. 24	Urine	P	*P. mirabilis*	>400	>400	200	50	50	25	12.5
M. I.	29	Feb. 13	Sinus tract	none	*P. mirabilis*	>400	>400	200	100	100	100	50
	10	Feb. 17	Sinus tract	none	*P. mirabilis*	>400	>400	200	100	25	50	12.5
P. R.	41	Feb. 9	Urine	P, T	*P. mirabilis*	>400	>400	100	100	50	50	25
	64	Feb. 23	Urine	P, T	*P. mirabilis*	>400	>400	200	100	50	50	12.5
P. M.	97	Mar. 16	Sputum	none	*P. mirabilis*	>400	>400	100	25	50	25	50
	98	Mar. 16	Feces	none	*P. mirabilis*	>400	>400	200	12.5	25	50	25

All of the strains grew in the maximum concentration (400 μg per ml.) of erythromycin, bacitracin, and polymyxin.
*P = Penicillin; Pm = Polymyxin; Cm = Chloramphenicol; T = Tetracycline; Ct = Chlortetracycline; S = Streptomycin.

morgani which exhibited swarming at 37° C. likewise reacted like the other strains of the same species which did not swarm, with the possible exception of the two swarmers among *P. vulgaris* which were the most susceptible of this species to chloramphenicol.

Comparison of Strains From Same Patient.—There were nine patients from each of whom two or three strains of Proteus were isolated, either from different sources at the same time or from the same or different sources at different times. The results of the tests for the susceptibility of these strains to various antibiotics are listed in Table V; since all strains were resistant to erythromycin, bacitracin, and polymyxin in the highest concentrations tested, these results are not listed. Unfortunately, there was only one patient, H. G., from whom strains of more than one species were isolated; a strain of *P. mirabilis* was cultured from the feces of this patient, while strains of *P. vulgaris* were obtained from the urine four days previously and eleven days later. Both of the strains of *P. vulgaris* were susceptible to each of the three tetracycline analogues, and resistant to streptomycin and penicillin; the strain of *P. mirabilis,* on the other hand, was resistant to the three tetracyclines and moderately resistant to streptomycin and penicillin, whereas all three strains were about equally susceptible to neomycin and chloramphenicol. All the strains from the other eight patients were *P. mirabilis* and resembled, in their susceptibility to various antibiotics, the strain of this species obtained from H. G., except that most of them were moderately susceptible to penicillin. In their susceptibility to penicillin, chloramphenicol, and streptomycin, there were some differences among strains obtained from different patients, but those isolated from the same patient were about equally susceptible to each of these antibiotics.

Comparison With Previous Results.—In the earlier study[6] of the antibiotic susceptibility of strains of Proteus isolated in 1949, seven antibiotics were used, including bacitracin, polymyxin B, and polymyxin D. The latter antibiotic is no longer available, and all of the strains tested were highly resistant to bacitracin and to polymyxin B, as they were in the present study. A comparison of the results obtained in the earlier study with the other four antibiotics, namely penicillin, streptomycin, chlortetracycline (Aureomycin), and chloramphenicol (Chloromycetin) and the results obtained with these antibiotics in the present study is shown in Table VI.

Since in the present study marked differences in antibiotic susceptibility were demonstrated among the species of Proteus, it is important to take this factor into account. Unfortunately, the strains in the earlier study had not been classified so that an adequate comparison of strains of the individual species in both series could not be made directly. However, twenty strains from the earlier study had been preserved in the lyophilized state. These twenty strains were reconstituted and subcultures were studied in the same manner as in the present study; they were classified into species by their biologic and biochemical reactions and tested for susceptibility to the ten antibiotics. One of the twenty strains was classified as *P. vulgaris,* one as *P. morgani,* and the other eighteen as *P. mirabilis.* The antibiotic susceptibilities of these strains were

comparable to those of the corresponding species of the present study, except that all of the earlier strains of *P. mirabilis* were inhibited by 200 μg. per milliliter or less of streptomycin and by 100 μg. per milliliter or less of penicillin.

Since the great preponderance of the 1949 strains appeared to be strains of *P. mirabilis,* the results for the recent strains of this species are also shown in Table VI. From the data available, there would appear to be little or no difference in the susceptibility to penicillin and streptomycin between the strains isolated in 1949 and those obtained in 1954, when due allowance is made for the probable differences in the distribution of the strains according to species. There is a suggestive increase in resistance to chloramphenicol in the recent strains, but the most striking difference revealed is in the greater resistance of the recent strains to chlortetracycline. In assessing the latter difference, however, the possibility of some change in the activity of the antibiotic resulting from refinements in manufacture[12] cannot be entirely excluded.

TABLE VI. SUSCEPTIBILITY OF STRAINS OF PROTEUS (ALL STRAINS) TO PENICILLIN, STREPTOMYCIN, CHLORTETRACYCLINE, AND CHLORAMPHENICOL: COMPARISON OF RESULTS OBTAINED IN 1949* AND IN 1954

ANTIBIOTIC	YEAR	PER CENT OF STRAINS INHIBITED BY: (MICROGRAMS PER MILLILITER)									NUMBER OF STRAINS TESTED
		3.1	6.3	12.5	25	50	100	200	400	>400	
Penicillin	1949	0	0	20	28	20	0	4	0	28	25
	1954, all	0	0.8	16.8	21.0	6.7	8.5	10.0	3.4	32.8	119
	P. mirabilis†	0	1	23	29	9	12	13	5	8	86
Streptomycin	1949	0	3.5	8.8	8.8	36.8	21.9	7.0	1.8	11.4	114
	1954, all	0	2.5	5.9	11.7	37.0	14.3	0.8	0	27.8	119
	P. mirabilis†	0	3.5	3.5	15	44	20	1	0	13	86
Chlortetracycline	1949	1.8	0	0.9	2.7	2.7	10.7	42.0	39.3	0	112
	1954, all	3.4	5.0	8.4	1.7	0.8	0.8	1.7	3.4	74.8	119
	P. mirabilis†	0	0	0	0	0	0	2	5	93	86
Chloramphenicol	1949	6	4	20	39	20	4	4	4	0	54
	1954, all	0	0.8	5.9	15.2	31.9	32.8	8.4	0.8	4.2	119
	P. mirabilis†	0	1	2	15	42	38	1	0	0	86

*Data for 1949 are from reference 6.
†1954.

Effect of Previous Antibiotic Therapy.—All the hospital records of the patients from whom strains of Proteus were obtained for the present study were reviewed for data concerning antibiotic therapy during the same or previous admissions. Such data are admittedly incomplete, except with respect to treatment actually given during the patient's stay at this hospital; negative data do not preclude antibiotic treatment at home or in other hospitals which were not elicited in the histories. The antibiotic susceptibilities of the strains obtained from patients who received antibiotics at any time before the strains of Proteus were obtained were compared with those of strains from patients with no evidence of prior antibiotic therapy. The antibiotics received before the isolation of the strains other than *P. mirabilis* are listed in Table III.

As far as could be ascertained, only three patients had previously received erythromycin, two had received both bacitracin and polymyxin, and none had been given neomycin; the results obtained with these four antibiotics, therefore, could not be considered related to therapy with any of these agents.

Chloramphenicol had been given to four patients in this study; from two of them, strains of *P. morgani* (Nos. 57 and 109, see Table III) were obtained and from the other two, there were isolated four strains of *P. mirabilis,* three of them from the same patient (E. M., Table V). Strain 57 was highly resistant to chloramphenicol and strain 109 was moderately resistant. The three strains from E. M. were equally susceptible (50 μg. per milliliter) but only the last of them was obtained after treatment with chloramphenicol. The remaining strain of *P. mirabilis* was inhibited by 100 μg. per milliliter. These values, therefore, fall into the general range of those for strains isolated from patients without prior exposure to this antibiotic.

Twelve strains were obtained from patients who had previously been treated with streptomycin; six of these strains were resistant to 400 μg. per milliliter of streptomycin. A similar degree of resistance to this antibiotic was noted in twenty-seven of the 107 strains from patients who presumably had no previous personal contact with streptomycin. Strains of Proteus resistant to streptomycin were, therefore, twice as frequent among those obtained from patients who had previously been treated with this antibiotic (50 per cent) as they were among strains isolated from patients who presumably had not previously received such treatment (25 per cent).

The pattern of distribution of the penicillin susceptibility of seventy-six strains of Proteus obtained from patients known to have previously received penicillin was almost identical with that of the forty-three strains isolated from patients in whom a history of such treatment could not be ascertained. The penicillin susceptibility of these strains appeared to be more clearly related to the species of the organisms, as already noted.

Since the strains of *P. mirabilis,* as a group, were almost all resistant to the three tetracyclines irrespective of prior treatment of the patient with any of these agents, only the strains of the other species need be considered. As can be seen from Table III, eight strains of *P. vulgaris, P. morgani,* or *P. rettgeri* were obtained from patients after treatment with any of the tetracycline analogues; four of these strains were resistant to these agents. The remaining twenty-five strains were from patients in whom there was no history of treatment with any of the tetracycline analogues; 5 of these strains were resistant. In this small group of strains of Proteus, therefore, strains resistant to the group of tetracycline antibiotics were two and one-half times as frequent among patients previously treated with these agents (50 per cent) as they were among patients who presumably had no previous personal experience with any of these antibiotics (20 per cent).

DISCUSSION

One of the objectives of the present study was to determine whether there has been an increase in the resistance of strains of Proteus to any of the anti-

biotics that have been available and widely used in recent years; another was to determine the relative in vitro activity of the more recently introduced antibiotics against these organisms. Data were presented concerning 119 strains of Proteus isolated from patients at the Boston City Hospital between December, 1953 and April, 1954; these strains were classified into species and tested for susceptibility to ten currently available antibiotics. The data thus obtained were then compared with the results of an earlier study in which strains of Proteus isolated in this hospital in 1949 were tested for susceptibility to six of the same antibiotics that were available at that time. Some of the comparisons were somewhat hampered by the fact that the earlier strains had not been classified according to species, but this was compensated for, in part, by a restudy of twenty of the earlier strains that were still available in the lyophilized state, which indicated that all but a small proportion of them were strains of *P. mirabilis.*

A definite and significant increase in resistance of the recent strains of Proteus could be demonstrated only against chlortetracycline; there was also a suggestive increase in resistance to chloramphenicol. No such increases in resistance to penicillin or streptomycin were discernible. All strains were resistant to the only other antibiotics that were used in both studies, namely bacitracin and polymyxin B, in the highest concentrations used.

Four new antibiotics not available in 1949 were used in the present study; these were oxytetracycline, tetracycline, neomycin, and erythromycin. The susceptibility of the recently isolated strains to these agents could be compared with their susceptibility to the older antibiotics. Oxytetracycline appeared to have essentially the same activity as chlortetracycline, but tetracycline was somewhat more active than either of its analogues when all 119 strains were considered together; this superiority of tetracycline, however, was limited to the strains of the most common species, namely *P. mirabilis.* Neomycin inhibited almost all of the strains in concentrations of 25 to 100 μg. per milliliter, being slightly more active *in vitro* than streptomycin and chloramphenicol against most of the strains. Erythromycin inhibited only a few of the strains in high concentrations, while most of them were quite resistant. The twenty strains from 1949 that were restudied included eighteen of *P. mirabilis,* and one each of *P. vulgaris* and *P. morgani*; their susceptibility to the ten antibiotics corresponded closely to that of the corresponding species of recently isolated strains.

Previous treatment of the patient with the homologous antibiotic did not appear to have any bearing on the resistance of the strains to erythromycin, bacitracin, polymyxin, or neomycin; these antibiotics, however, were seldom used in the patients from whom the strains were obtained. Treatment with penicillin at any time prior to the isolation of the strains did not seem to be a factor in the results of the tests with penicillin since the proportion of penicillin-resistant strains was similar among patients who had received this antibiotic and among those who had not. The data for chloramphenicol were inadequate in this respect. Treatment with any of the three tetracycline analogues appeared to be responsible for an increase in the proportion of strains of Proteus resistant

to all three agents of this group; this increased resistance to the tetracyclines, however, was discernible only among the species other than *P. mirabilis.* A significant increase in streptomycin-resistant strains was also noted among those isolated from patients who had been treated with this antibiotic at any time prior to the isolation of the strains.

The most interesting and important observation was the correlation between certain of the biochemical reactions of strains of Proteus which characterize the various species and the susceptibility of these strains to some of the antibiotics. In this respect, *P. mirabilis* appeared to behave differently from the other three species that were studied, namely, *P. vulgaris, P. morgani,* and *P. rettgeri.* This was most clearly discernible with respect to penicillin and the three tetracycline antibiotics. Strains of *P. mirabilis* varied in their susceptibility to penicillin but most of them were inhibited by concentrations ranging from 12.5 to 200 μg. per milliliter; strains of the other three species, all of which were indole producers, were almost all resistant to higher concentrations. The reverse was true with respect to the tetracyclines; the strains of *P. mirabilis* were almost all quite resistant to each of these agents, although somewhat less so to tetracycline than to chlortetracycline or to oxytetracycline, whereas the strains of *P. vulgaris* were all quite susceptible and those of *P. morgani* and *P. rettgeri* varied, some being susceptible and others resistant. The strains of *P. mirabilis* also appeared to be somewhat more susceptible to streptomycin and to chloramphenicol as compared with the strains of other species, but the differences were not so striking.

No relationship between swarming and susceptibility to antibiotics could be discerned beyond that which is related to the species of the strain; the data on this aspect, however, are inadequate.

Kylin and Lindberg[13] demonstrated a statistically significant correlation between positive indole and methyl red reactions of coliform bacteria and susceptibility to sulfamethylpyrimidine, streptomycin, chloramphenicol, and oxytetracycline. On the other hand, positive Voges-Proskauer, inositol, and citrate reactions were associated with increased resistance to antibiotics. Chlortetracycline, however, behaved differently from the other agents and did not show such correlations. The data, however, are not very convincing and the differences noted were not striking.

From the point of view of therapy, the observations reported here suggest that the test for production of indole may be the most useful one to apply to strains of Proteus as an aid in the choice of the appropriate antibiotic. Judging only from these in vitro results, penicillin (in very large doses[14]), streptomycin, chloramphenicol, or neomycin would be the antibiotics of choice for infections with strains which do not produce indole (classified as *P. mirabilis*), and tetracycline should also be effective in infections of the urinary tract with such strains. For the treatment of infections with indole-producing strains of Proteus, especially strains of *P. vulgaris,* any of the three tetracyclines would appear to be the antibiotics of choice, and neomycin would seem to be as effective against any of these organisms as against strains of *P. mirabilis.* The toxicity

of neomycin when used parenterally may preclude its use in most systemic infections or in infections of the urinary tract; however, its poor absorption when given orally suggests that neomycin may be preferred for Proteus infections within the intestinal tract, such as have occasionally been reported.[15]

The patients from whom the strains used in this study were obtained were not treated according to protocols which would permit proper evaluation of the comparative effectiveness of the antibiotics, or to correlate the in vivo effects with the in vitro results. Waisbren and Carr[1] have found a combination of penicillin and chloramphenicol to be effective in some cases but not in others, and the results of treatment did not always correspond with those of the in vitro tests. Yow[2] found streptomycin and sulfonamides to be the most effective treatment of Proteus infections. The action of antibiotic combinations was not included in the present study.

SUMMARY AND CONCLUSIONS

One hundred and nineteen strains of Proteus isolated from patients at the Boston City Hospital between December, 1953, and April, 1954, were characterized as to their species and tested for their in vitro susceptibility to ten antibiotics. Included were eighty-six strains of *P. mirabilis,* twelve of *P. vulgaris,* fifteen of *P. morgani,* and six of *P. rettgeri.*

The susceptibility of the strains of Proteus to the tetracycline antibiotics varied with the species; strains of *P. mirabilis* were moderately to highly resistant to chlortetracycline and to oxytetracycline and appreciably more susceptible to tetracycline. The strains of *P. vulgaris* were susceptible, and equally so, to all three analogues and those of *P. morgani* and *P. rettgeri* varied, some being susceptible and others resistant to all three of these tetracycline antibiotics.

The susceptibility of the strains to penicillin also was related to the species, those of *P. mirabilis* varied from moderately susceptible to highly resistant, while those of the other three species were all resistant.

The strains of *P. mirabilis* appeared to be somewhat more susceptible to streptomycin and chloramphenicol than those of the other three species, which included a greater proportion of strains that were highly resistant to these two antibiotics.

Neomycin inhibited almost all strains of all species in concentrations of 25 to 100 μg. per milliliter, being slightly more active in vitro than streptomycin and chloramphenicol against most of the strains. Erythromycin inhibited only a few strains in high concentrations and most of the others were quite resistant to this agent. All strains were resistant to bacitracin and polymyxin B.

A comparison of the present findings with the results obtained in 1949 showed no definite increase in resistance of Proteus to penicillin or streptomycin, possibly some increase in resistance to chloramphenicol, but a definite and significant increase in the proportion of strains resistant to chlortetracycline.

Previous treatment of patients with penicillin did not appear to influence the susceptibility to this antibiotic of stains of Proteus subsequently isolated from such patients; streptomycin-resistant strains, however, were more frequent

among patients previously treated with this agent than among those who had not received streptomycin before the strains were isolated. Strains of Proteus isolated from patients any time after they had been treated with any of the tetracycline analogues included a larger proportion that were resistant to these three antibiotics when compared with strains obtained from patients not previously treated with any of the tetracycline antibiotics.

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LABORATORY METHODS

BACTERIAL β-GLUCURONIDASE AS A REAGENT FOR THE HYDROLYSIS OF URINARY CORTICOSTEROIDS

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IN THE determination of urinary steroids, a major problem is the hydrolysis of the conjugated forms. The usual methods for hydrolyzing these forms have employed relatively severe treatment with strong acid which is known to result in structural changes of some of the steroids.[2-4] A less destructive method of hydrolysis makes use of enzyme action. Since many of the steroids are excreted in the urine as glucuronides, β-glucuronidase may be used to split these conjugates.

Cohen and Marrian[5] observed that exposure of pregnancy urine to prolonged bacterial action resulted in more complete hydrolysis of the conjugated estrogens. This hydrolysis was later shown to involve a bacterial glucuronidase.[6] Subsequently, preparations of glucuronidase from animal sources were used for the hydrolysis of C19 and C21 steroid glucuronides.[7-12] Buehler and associates[13] reported the successful application of a bacterial glucuronidase preparation for the efficient hydrolysis of steroid glucuronides. Using a reduction method for the determination of steroids and an enzyme preparation from *Escherichia coli,* Kinsella and colleagues[14, 15] showed that the yields of extractable adrenal corticosteroids from urine could be considerably increased.

Since most of the quantitative studies on the use of glucuronidase for hydrolysis of steroids have been made with preparations from animal sources,[9-12] the ready availability of a potent bacterial preparation made it advisable to investigate this material as a possible tool in the quantitative determination of urinary adrenal cortical hormones and their metabolites in urine. Determination of these substances has become increasingly important during the past few years for studying not only the endocrine activity of the adrenal cortex in health and disease, but also the effects of stress and ACTH administration on this activity.

In a recent conference[16] on estimation of steroids in blood and urine, it became apparent that a routine method of enzymatic hydrolysis would be of value. Methods using spleen β-glucuronidase[9-12] require high concentrations of the enzyme. This limits its use to those laboratories where large amounts of the spleen β-glucuronidase presents no problem. A method suitable for routine analysis of adrenal corticosteroids using bacterial β-glucuronidase is presented in this report. This requires considerably less enzyme concentration than the ones using spleen β-glucuronidase. Comparison of this method with one using a spleen preparation*[11] was made. The apparent advantages of the former method are: speed, economy, since less material of the bacterial preparation is required, and less background color resulting during incubation, since hydrolysis takes place at neutrality.

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This investigation was supported by research grants from the American Cancer Society and the Office of Naval Research under contract Nonr. 1035 (00). A preliminary report of this work was presented at the meeting of the American Society of Biological Chemists at Chicago, April 6-10, 1953 (1).

Received for publication Feb. 15, 1954.

*The author wishes to thank Drs. H. P. Dustan, H. L. Mason, and A. C. Corcoran for making available to him, prior to publication, their method for enzyme hydrolysis using a spleen preparation.

MATERIALS AND METHODS

Enzyme.—Bacterial β-glucuronidase* and spleen β-glucuronidase.† The bacterial β-glucuronidase solution is freshly prepared by dissolving the β-glucuronidase in distilled water to yield a concentration of 500 units‡ per milliliter. The spleen β-glucuronidase was prepared according to Dustan and associates.[11]

0.3M phosphate buffer, pH 7.00
1.0M acetate buffer, pH 4.50
2N NaOH
0.1N NaOH
Concentrated HCl
$HCCl_3$, C. P.

Periodic acid reagent, $SnCl_2$ and chromotropic acid reagent as required in formaldehydogenic corticosteroid determination of Corcoran and Page.[17]

Reagents for carrying out the Porter-Silber reaction as described.[18]

A twenty-five milliliter aliquot of urine is adjusted to pH 7.0 with either 2N sodium hydroxide or concentrated hydrochloric acid. To this is added 5 ml. of 0.3M phosphate buffer (pH 7.00), 1 ml. of a bacterial β-glucuronidase solution containing 500 units, and 5 ml. of chloroform. After the mixture has been incubated at 38° C. for from twenty to twenty-four hours, it is removed from the incubator and adjusted to pH 1.0 with concentrated hydrochloric acid. The mixture is extracted four times with 25 ml. chloroform each time. The chloroform extracts are combined and anhydrous sodium sulfate is added to break the emulsion. The clarified chloroform is filtered and extracted two times with 12 ml. 0.1N sodium hydroxide each time and then once with 12 ml. water. The combined sodium hydroxide and water washings are re-extracted with 25 ml. chloroform. The combined chloroform extracts are dried over anhydrous sodium sulfate and evaporated to dryness under vacuum at a temperature less than 45° C. The formaldehydogenic corticosteroids present in the residue are determined according to the method of Corcoran and Page.[17] A blank in which water is substituted for urine but which contains all other reagents, including enzyme, is incubated and extracted along with the urine samples. A single lot of chloroform is employed for each set of analyses. A corrected value is obtained by subtracting the value of formaldehydogenic material obtained for the blank from that obtained for the urine. The spleen preparation was employed according to the method of procedure 1 of Dustan and associates.[11] The phenylhydrazine-H_2SO_4 reaction was carried out according to the method of Porter-Silber.[18]

RESULTS AND DISCUSSION

Fig. 1 presents the influence of pH on the hydrolysis of formaldehydogenic steroid glucuronides with bacterial β-glucuronidase. The ordinate is expressed as the per cent of the amount of formaldehydogenic corticosteroids obtained prior to the enzyme hydrolysis. The points represent the averages of several determinations.

The optimum pH for this hydrolysis was near 7.0. Although there is a decline of hydrolytic activity on both sides of this point, the drop on the alkaline side was much more pronounced than on the acid side. The optimum of pH 7.0

*Purchased from Sigma Chemical Co., St. Louis, Mo.

†Purchased from Viobin Corporation, Monticello, Ill.

‡One unit of bacterial β-glucuronidase activity is that amount of enzyme which will liberate one microgram of phenolphthalein from phenolphthalein glucuronide in one hour under the following conditions: pH 6.0 (0.05M Acetate buffer), phenolphthalein glucuronide concentration between 0.0003 and 0.0005M, incubation temperature of 37° C. The spleen β-glucuronide is assayed at pH 4.5.

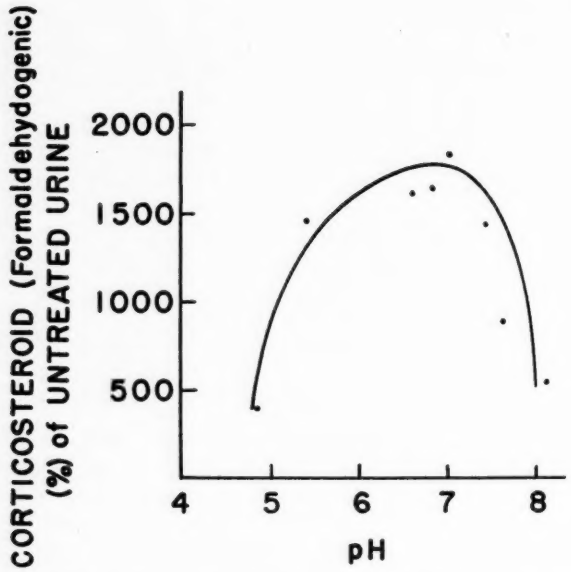

Fig. 1.—Effect of pH on release of corticosteroid by bacterial β-glucuronidase. Per cent of untreated urine $= \frac{\text{mg. corticosteroid after enzyme hydrolysis}}{\text{mg. corticosteroid before enzyme hydrolysis}} \times 100$. Five hundred units of bacterial β-glucuronidase per 25 ml. urine and an incubation time of 20 to 24 hours employed.

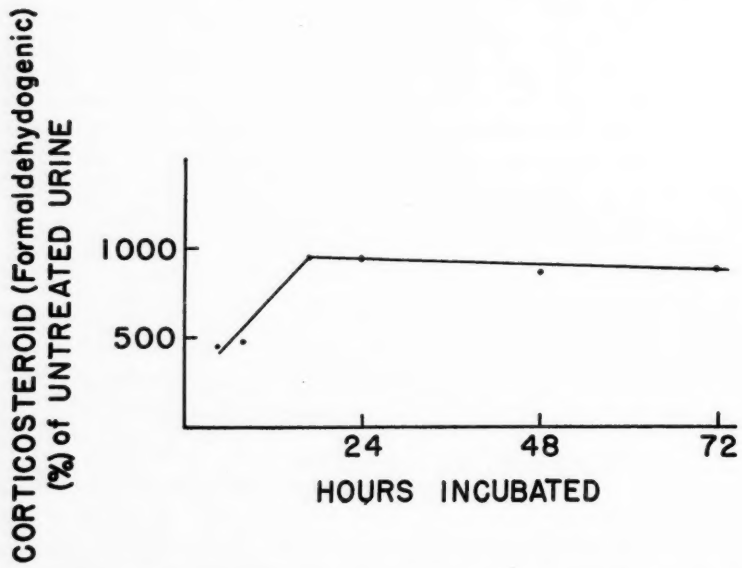

Fig. 2.—Influence of incubation time on release of corticosteroid by bacterial β-glucuronidase, concentration of 500 units per 25 ml. urine, pH 7.0. Per cent of untreated urine $= \frac{\text{mg. corticosteroid after enzyme hydrolysis}}{\text{mg. corticosteroid before enzyme hydrolysis}} \times 100$.

has the advantage of resulting in the formation of less nonspecific chromogenic material. The spleen preparation has an optimum pH of 4.5.[11]

The amount of formaldehydogenic material determined in the reagent blank using the bacterial preparation at pH 7.0 was less than 0.010 mg. per 25 ml. sample and usually ranged from 0.000 to 0.005 mg. The spleen preparation resulted in reagent blanks which usually yielded more than 0.020 mg. of formaldehydogenic material.

Fig. 2 illustrates that the enzymatic hydrolysis was complete after eighteen hours and remained virtually unchanged up to seventy-two hours, which was the longest incubation time studied. To establish whether the enzyme may have been inactivated in this time, a second incubation was carried out after the addition of more bacterial β-glucuronidase. No more formaldehydogenic material was obtained. The hydrolysis may have been complete before the eighteen-hour-period since determinations between eight and eighteen hours were not made. For the sake of convenience we incubated for about twenty-four hours. An incubation period of seventy-two hours has been recommended for the spleen preparation.[11]

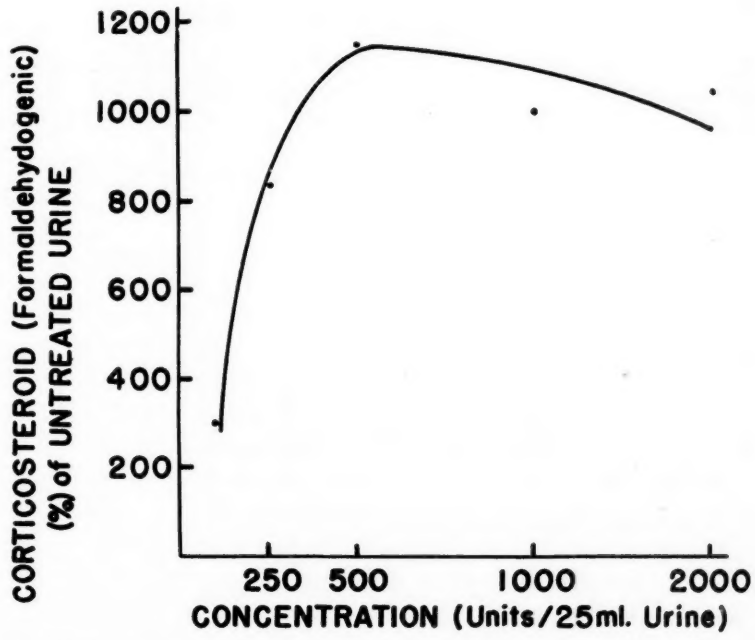

Fig. 3.—Effect of concentration of bacterial β-glucuronidase on release of corticosteroid. Per cent of untreated urine = $\frac{\text{mg. corticosteroid after enzyme hydrolysis}}{\text{mg. corticosteroid before enzyme hydrolysis}} \times 100$. Incubation time was 24 hours.

The influence of enzyme concentration on the release of formaldehydogenic substances is depicted in Fig. 3. The time for hydrolysis was twenty-four hours. To eliminate the possible variable inhibitory activity that different urines might have, aliquots of the same urine were used to determine the

amount of formaldehydogenic material liberated at the different enzyme concentrations. The points represented by the curve are averages of from five to nine determinations.

Fig. 4 represents data which compare the amount of formaldehydogenic material released by the method outlined previously using the bacterial β-glucuronidase with that obtained by the method of Dustan and others[11] using the spleen preparation. Pooled urines were used. The bars represent averages obtained for the same pooled urine and each group represents a different urine. The results are given as milligrams of corticosteroid per 25 ml. of urine. In urine 1 the method using the spleen preparation was somewhat more effective. In urines 2 and 5 the bacterial enzyme was better. There was no appreciable difference in urines 3 and 4. Even though fewer units of the bacterial enzyme preparation was used and the time for incubation was shorter, the yield of formaldehydogenic material was at least equal to that obtained with the method of Dustan and colleagues,[11] using the spleen preparation.

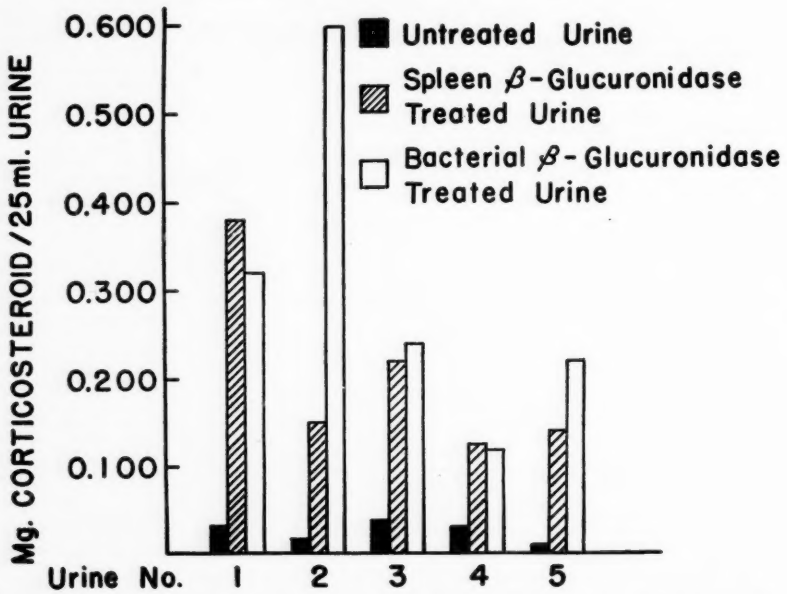

Fig. 4.—Comparison of spleen and bacterial β-glucuronidase on the release of corticosteroid. The spleen β-glucuronidase used according to Dustan and associates,[11] while the bacterial β-glucuronidase was used according to method in text.

There has been some question raised as to whether the material which is measured after enzymatic hydrolysis is in effect an indication of adrenal cortical activity.[19] The data of Table I provide evidence that there is a correlation between the amount of formaldehydogenic corticosteroid extracted both before and after enzymatic hydrolysis and the adrenal state. The table includes conditions representing both hyper- and hypo-adrenal states. In ten additional patients who exhibited hypoadrenalism either due to Addison's disease or due to pituitary failure, none gave large amounts of formaldehydogenic material after enzymatic hydrolysis. In this laboratory, the highest amount of formalde-

TABLE I. CORTICOSTEROIDS EXTRACTABLE FROM URINE REPRESENTING DIFFERENT ADRENAL STATES BEFORE AND AFTER TREATMENT WITH BACTERIAL β-GLUCURONIDASE

URINE DESIGNATION	ADRENAL STATE	FORMALDEHYDOGENIC CORTICOSTEROIDS (MG./24 HOURS)	
		BEFORE ENZYMATIC HYDROLYSIS (a)	AFTER HYDROLYSIS WITH BACTERIAL β-GLUCURONIDASE (b)
4049 W.T.	Receiving ACTH	3.36	34.70
4371 M.N.	Cushings	1.30	19.30
5104 N.B.	Adrenal cortical hyperplasia, with virilism	0.64	20.85
5174 K.B.	Normal	0.36	3.95
5244 H.J.	Panhypopituitarism	0.05	1.33

Values in (a) obtained by method of Corcoran, and Page.[17] Values in (b) obtained by method presented in this paper.

hydogenic material obtained after enzymatic hydrolysis in patients with hypoadrenalcortical function has been 2.69 mg. per twenty-four hours. We consider a value of 3.00 mg. per twenty-four hours with this procedure as the lower limit of normal.

TABLE II. THE EFFECT OF BACTERIAL β-GLUCURONIDASE ON URINARY CORTICOSTEROIDS AS MEASURED BY THE FORMALDEHYDOGENIC AND PORTER-SILBER REACTIONS

URINE DESIGNATION	FORMALDEHYDOGENIC CORTICOSTEROID (MG./24 HOURS)		Ø HYDRAZINE H_2SO_4 CORTICOSTEROID (MG./24 HOURS)	
	BEFORE ENZYMATIC HYDROLYSIS	AFTER HYDROLYSIS WITH BACTERIAL β-GLUCURONIDASE	BEFORE ENZYMATIC HYDROLYSIS	AFTER HYDROLYSIS WITH BACTERIAL β-GLUCURONIDASE
3169 A.L.	2.30	6.35	0.65	3.30
5017 A.S.	0.84	4.84	0.96	3.23
5021 M.C.	3.38	8.60	1.76	7.07
5061 H.McC.	4.18	13.20	3.75	9.00
5065 J.L.	0.82	2.06	1.08	3.10
5089 A.S.	1.63	6.85	1.07	6.28
5127 F.G.	0.67	12.04	0.26	3.38
5172 J.DaV.	1.44	5.31	5.84	13.59

Further evidence that the increase which is being measured after enzymatic hydrolysis is adrenal cortical material is given in Table II. An additional criterion for excretion of these steroids is utilized. This is the Porter-Silber reaction[18] which is specific for 17,21-dihydroxy, 20 ketosteroids. Although it is recognized that this method is not too satisfactory when applied to relatively crude urine extracts because of the high sulfuric acid blanks which result, it will be seen that the amounts of corticosteroids excreted in the urine measured by the Porter-Silber reaction as well as the formaldehydogenic method were increased after enzymatic hydrolysis. The difference in values determined by these two methods may, in part, be due to the fact that different chemical groups are being measured. The formaldehydogenic steroids are a measure of the α-ketolic group, whereas the Porter-Silber reaction is specific for the 17,21-dihydroxy, 20-keto group. However, both these determinations are influenced in the same direction after enzymatic hydrolysis. Kinsella and Glick[15] have presented evidence which indicates that the larger values of neu-

tral reducing lipids obtained after treating urines with β-glucuronidase of bacterial origin may be related to adrenal secretory activity. The urines presented in Table II are from random patients. It is interesting to note that urine 5065, J. L., which gave a low formaldehydogenic corticosteroid value after enzymatic hydrolysis was from an Addisonian patient.

In order to determine whether the bacterial β-glucuronidase preparation has a destructive influence on known adrenal cortical steroids, both compound E and compound F were incubated with the preparation and recovery experiments conducted. Aqueous aliquots equivalent to 0.125 mg. per 25 ml. of each of these compounds were treated with the preparation in the manner described previously. It was possible to recover an average of 91 per cent of compound E and 92 per cent of compound F from the incubation mixture after treatment with bacterial β-glucuronidase. The quantitative determinations were made by both the formaldehydogenic and Porter-Silber techniques.

SUMMARY

A procedure using bacterial β-glucuronidase as a hydrolytic agent for the determination of urinary adrenal cortical steroids is presented. The method is simple, quick, and economical, and the results obtained correlate well with the adrenal status. Five hundred units of the bacterial β-glucuronidase and an incubation time of twenty-four hours give results comparable to those obtained with 7,500 units of the spleen β-glucuronidase and an incubation time of seventy-two hours. There is no significant destruction of compound E or compound F when they are incubated with bacterial β-glucuronidase.

We gratefully acknowledge the assistance of Mrs. Marylin Musgrave in conducting many of the technical procedures.

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THE RAPID IDENTIFICATION OF BILE ACIDS WITH AN ANTIMONY TRICHLORIDE COLOR REAGENT

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INTRODUCTION

CHROMATOGRAPHY[2, 3, 5] and counter current[1] distribution have provided valuable tools for the isolation and partial identification of bile acids in biologic material. Additional procedures, such as spectrophotometry, are, however, frequently required for complete identification. While searching for methods which would permit detection of adrenocorticoids on paper chromatograms, Neher and Wettstein[4] found that microgram quantities of cholic and desoxycholic acids applied to paper gave color reactions with the Carr-Price (antimony trichloride) reagent. We have studied the reaction of twelve bile acids with antimony trichloride in a spot test and have found it valuable for the identification, characterization, and quantitative approximation of these substances.

MATERIALS AND METHODS

A solution of antimony trichloride in chloroform, saturated at room temperature, is prepared by adding about 800 ml. analytic reagent grade chloroform to one-half pound of crystalline analytic reagent grade antimony trichloride in a dry glass bottle of about one liter capacity, provided with a tightly fitting glass stopper. The bottle is shaken occasionally and the solution permitted to come to equilibrium at room temperature over a period of seven to ten days. It is considered saturated and ready for use when no more schlieren formation can be detected on gentle shaking in the presence of crystalline antimony trichloride. The reagent is readily decomposed by water or alcohols. The solution should be clear and free of any turbidity; the latter indicates decomposition and is usually caused by water which may come from the atmosphere through a leaking stopper. If moisture and alcohols are excluded, the reagent is stable for months. Insoluble oxychlorides and hydroxides which are likely to form at the mouth of the bottle should be wiped off before the reagent is poured. Clean dry bottles should be used for the preparation of new batches of reagent, since decomposition products of antimony trichloride interfere with the spot test. These decomposition products may be removed from glassware with concentrated hydrochloric acid.

Standard solutions of bile acids in acetone (redistilled) containing 1 mg. per milliliter are applied to Whatman No. 1 filter paper with micro pipettes. The resulting circular spot is limited to a diameter of 7 to 10 mm. After the bile acids are applied the paper is dried in an oven at 90° C. for four minutes. During the last minute of drying, antimony trichloride is poured into a shallow dish to a depth of about 0.5 cm. The paper is removed from the oven, rapidly dipped in the solution, and returned to the oven without delay for an additional four and one-half minutes. The colored spots are observed with an ultraviolet lamp (emission peak 365 mμ*). If rectangular pieces of paper are used, the ends may be conveniently brought together and fastened with a paper clip, forming a cylinder which can be placed on end in the oven, thus avoiding contacts with surfaces which tend to discolor the paper. Once used, the antimony trichloride reagent must be discarded since rapid decomposition occurs with room air; the rate of decomposition increases with the humidity.

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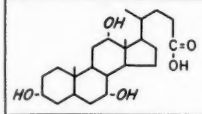
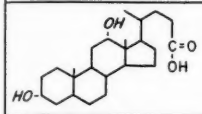
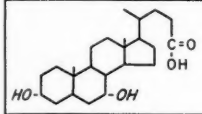
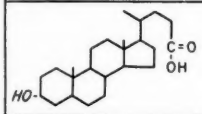
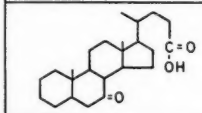
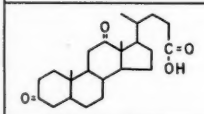
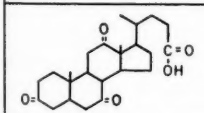
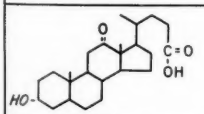
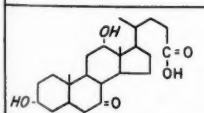
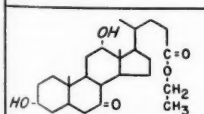
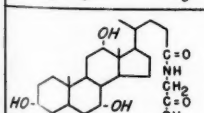
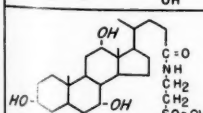
Sponsored by the Commission on Liver Disease, Armed Forces Epidemiological Board, and supported in part by the Office of the Surgeon General, Department of the Army.

Received for publication April 30, 1954.

*Black Raymaster, Type B. George W. Gates and Company, Long Island, N. Y.

TABLE I.—THE COLOR OF FLUORESCENCE PRODUCED IN ULTRAVIOLET LIGHT WITH VARYING AMOUNTS OF TWELVE SEPARATE BILE ACIDS

Bile Acids	Fluorescence and Quantities				
	5 γ	10 γ	20 γ	40 γ	80 γ
 3,7,12-trihydroxycholanic acid (cholic acid)	peach + +	peach + + +	peach + + +	rust + + +	dark rust + + + +
 3,12-dihydroxycholanic acid (desoxycholic acid)	pink + +	pink + + +	pink + + +	yellow + + +	bright yellow + + + +
 3,7-dihydroxycholanic acid (chenodesoxycholic acid)	purple +	purple +	purple + +	purple + + +	purple + + + +
 3-hydroxycholanic acid (lithocholic acid)	rose +	rose + +	rose + +	rose + + +	rose + + + +
 7-ketocholanic acid	blue +	blue + +	blue + + +	pale yellow + + +	pale yellow + + + +
 3,12-diketocholanic acid	violet +	violet +	red-violet + +	red-violet + + +	red-violet + + + +
 3,7,12-triketocholanic acid	blue-violet +	blue-violet + +	blue-violet + +	blue-violet + + +	blue-violet + + + +
 3-hydroxy, 12-keto-cholanic acid	violet +	violet +	red-violet + +	red-violet + + +	yellow-violet + + +
 3,12-dihydroxy, 7-keto-cholanic acid	blue +	blue + +	light peach +	light peach + +	light peach + + + +
 ethyl 3,12-dihydroxy–7-ketocholanate	blue +	blue + +	light peach +	light peach + +	light peach + + + +
 glycocholic acid	peach + +	peach + + +	peach + + +	dark peach + + +	dark peach + + + +
 taurocholic acid	peach + +	peach + + +	peach + + +	dark peach + + +	dark peach + + + +

RESULTS AND DISCUSSION

Table I lists the color of the fluorescence produced in ultraviolet light with 5, 10, 20, 40, and 80 μg amounts of twelve separate bile acids. The number of plus marks below the fluorescence descriptions indicate relative intensities. The cholanic acids with one, two, or three hydroxyl groups are easily distinguished from each other in amounts of 5 μg. The difference in fluorescence produced by two isomeric compounds, 3, 12 dihydroxycholanic acid and 3, 7 dihydroxycholanic acid, is distinctive, indicating that the type of fluorescence is related not only to the number of hydroxyl groups on the steroid ring but also to their position. Keto-acids can readily be differentiated from the hydroxy acids and from each other in 5 μg amounts. Cholanic acids containing both keto- and hydroxyl groups require slightly larger amounts ranging from 20 to 80 μg, for proper differentiation. Of the nine unconjugated bile acids tested, each gave a different and characteristic fluorescent color. Substitution on the carboxyl group did not appreciably alter the fluorescence produced by the parent compound. Taurocholic acid and glycocholic acid gave essentially the same fluorescent color as cholic acid; minor differences occurred with 40 μg quantities or more. The fluorescence of 3, 12 dihydroxy 7 ketocholanic acid and its ethyl ester were identical.

The shade and intensity of the spots change according to the amount of bile acid present. This feature permits semiquantitative estimations. The fluorescence descriptions, being subjective, will vary somewhat among observers and with the emission peak of the ultraviolet lamp used. Descriptions are reported here only to emphasize the difference in fluorescence of the various bile acids. The spots can also be seen in white light but the colors are not as distinctive nor as brilliant as the fluorescence in ultraviolet light. Bile acids may be applied to the paper in solutions of benzene, alcohol, petroleum ether, and isopropyl ether without alterations of fluorescence. The papers on which the spot tests are performed may be preserved as experimental records since the fluorescence loses little of its character or intensity over a period of months.

SUMMARY

Bile acids can rapidly be identified and approximately quantitated by a spot test using a saturated solution of antimony trichloride in chloroform. The fluorescence produced is specific for the position and number of hydroxyl or keto-groups attached to the cholanic acid nucleus. It changes qualitatively and quantitatively with the concentration of the bile acids. Amino or ethyl groups attached to the carboxyl group of the side chain do not appreciably influence the fluorescence.

We gratefully acknowledge the interest and encouragement of Dr. C. J. Watson and Dr. Samuel Schwartz, and the excellent technical assistance of Miss Betty Oseid. Dr. Erwin Mosbach of Columbia University kindly supplied the Chenodesoxycholic acid used in this study. We are also indebted to Dr. M. L. Tainter, of The Sterling-Winthrop Research Institute, Dr. Max Tishler of Merck and Company, Inc., Carl J. Mercer and J. B. Lesh of the Bio Process Company, Division of The Armour Laboratories, and Mr. Paul de Haen of Ames Company, Inc., for their valuable and generous contributions of bile acids.

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AN APPARATUS FOR RECORDING ARTERIAL AND VENOUS BLOOD PRESSURES ON A DIRECT-WRITING ELECTROCARDIOGRAPH

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Bethesda, Md.

A DEPENDABLE, inexpensive, easily portable apparatus is described which, when connected to any direct-writing electrocardiograph, will produce accurate pressure records. The pressure pickup is a commercially available strain gauge type, modified to provide a pressure base line on the electrocardiograph. No modification of the electrocardiograph is required, connections to it being made through the usual patient cable.

METHODS AND MATERIALS

The Statham Physiological Strain Gauge Types P-23A and P-23B with the associated control box Type CB-7 produce an electrical output voltage which is of the correct magnitude for connection to the patient leads of any direct-writing electrocardiograph. The pressure record thus produced will straddle the zero signal position of the electrocardiograph stylus. It will be possible to obtain from this record the pulse pressure, i.e., the difference between systolic and diastolic pressure, and the wave form of the pressure, but it will be impossible to obtain the absolute value of any pressure point on the record. The reason for this is that a pressure wave consists of an average or "integrated mean" value plus a complex alternating component. The electrical voltage produced by this pressure on the gauge thus contains a d.c. component proportional to the average pressure and a complex a.c. component proportional to the alternating component of pressure. If this voltage is impressed upon the input terminals of a standard direct-writing electrocardiograph, the first RC coupling network will pass only the a.c. component. The d.c. component will appear and be preserved as a potential difference between the terminals of the coupling capacitor. Thus the record produced is of the a.c. component only. This tracing will be of the correct shape, but will bear the same relationship to the zero input position of the electrocardiograph stylus that the true pressure curve bears to the average value. In other words, the coupling network has, in effect, made the average value zero but otherwise has not altered the input wave. In order to calibrate this tracing, it is necessary to find a means to reinsert the average value.

Consider the typical RC coupling network of Fig. 1. Here the RC product is large compared to the time between heartbeats. The input voltage E_i comes from the pressure gauge and therefore contains the components E_{ac} and E_{av} discussed previously. When the switch SW is in the R or "record" position as shown, E_{ac} appears at the output, while E_{av} appears across the capacitor with the polarity indicated. The output $E_o = E_{ac}$ connects to the e.c.g. and is recorded. If the switch is now thrown quickly to A, the "average" position, the output voltage is initially $-E_{av}$, decaying exponentially to zero. This voltage will deflect the electrocardiograph stylus downward instantaneously an amount proportional to the average pressure. The stylus will return to its zero signal position in a brief period. The true values for the average, systolic, and diastolic pressures are determined from the appropriate standardization deflection. This is described in detail below.

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Received for publication May 23, 1954.

Fig. 2 is a photograph of the complete apparatus. The Statham pressure gauge is in the right foreground. It connects by means of a flexible cable to the control box. The calibration switch in the right upper corner of the control box selects one of three standardizing resistors. This switch knob is labeled *50 A, 20 B,* and *5 B.* When the switch is in the *50 A* position, momentary depression of the standardize push switch will deflect the electrocardiograph pen downward the same amount as the sudden release of 50 Hg from the gauge. The sensitivity control of the electrocardiograph is adjusted to provide a convenient deflection of the stylus corresponding to the 50 mm. Hg. The letter *A* indicates that the P-23 A arterial pressure gauge is to be used for this range. The other two positions are for use with the P-23 B gauge and will give 20 mm. Hg and 5 mm. Hg standardization deflections. The three standardizing resistors must be adjusted to the proper value for the individual pressure gauges which are to be used.* The zero check push switch, which is located in the center of the control box, is used to balance the bridge for zero pressure. This switch is pressed and released while adjusting the zero adjust potentiometer. At the balance point the stylus of the electrocardiograph will not deflect when the zero check switch is pressed. The control box is fitted with five binding posts which are color coded to correspond to the five patient leads of the electrocardiograph. The lead selector switch of the electrocardiograph is positioned on Lead I, thus permitting the pressure signals to arrive at the recording stylus.

RESULTS

In Fig. 3 are pressure recordings from the right ventricle, right auricle, and aorta of an anesthetized dog. The right ventricular and right auricular tracings were made by cardiac catheterization via the femoral vein with a small polythene catheter. The electrocardiograph in this case is the Sanborn Viso Cardiette Model 51. The negative pulse at the left of each of the records is the calibration deflection obtained by pressing the standardize switch with the range selection switch in the position indicated for each of the tracings. In each case the pressure tracings straddle the zero input position of the stylus. The flat-bottom negative excursions seen in the pressure records result from throwing the Record-Average switch to the Average position. The vertical distance on the records from the zero signal input position to the maximum negative excursion obtained by throwing the Record-Average switch to the Average position is proportional to the average or "integrated mean" pressure. The constant of proportionality for this excursion is the same as the constant for the pressure record and is obtained from the calibration deflection. In the aortic pressure tracing, for example, each millimeter vertical division of the calibration deflection represents 5 mm. Hg pressure. Since the systolic-diastolic excursion is 14 mm., the pulse pressure is $14 \times 5 = 70$ mm. Hg. The average pressure excursion is 25 mm. below the zero input position, and the mean pressure is $25 \times 5 = 125$ mm. Hg. The diastolic pressure is 3 mm. below the zero input position or 15 mm. Hg below the mean pressure. Thus the diastolic pressure is 110 mm. Hg. The systolic pressure is 11 mm. above the zero input position, or 55 mm. Hg above the mean pressure. Thus the systolic pressure is 180 mm. Hg. As a check on the calculations, the pulse pressure is $180 - 110 = 70$ mm. Hg, which we previously obtained by a different method. Since the pressure scale is linear, it is permissible to add a pressure scale to the record based on

*A complete circuit diagram and description of the method by which the standardization resistors are determined can be obtained from Mr. F. W. Noble, National Institutes of Health, Bethesda, Md.

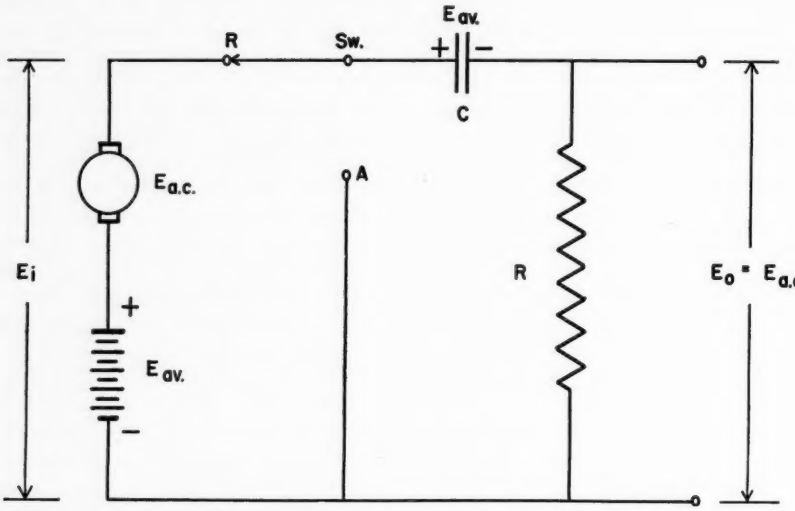

Fig. 1.—A simplified electrical circuit illustrating the method for reinserting the average pressure value. (See text for explanation.)

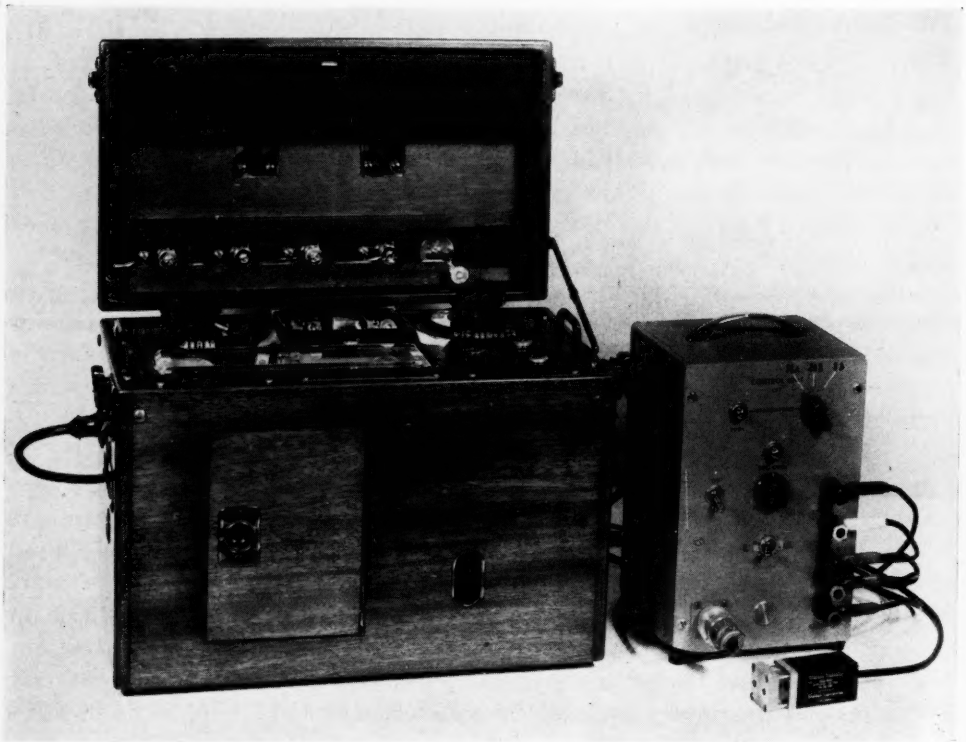

Fig. 2.—A photograph of the complete pressure recording apparatus. The Statham gauge is in the right foreground, the control box just behind, and the direct-writing ECG is on the left.

the calculated systolic and diastolic values. This will facilitate the determina- of any intermediate values of pressure and will also bring the record into conformity with the standard style of tracings.

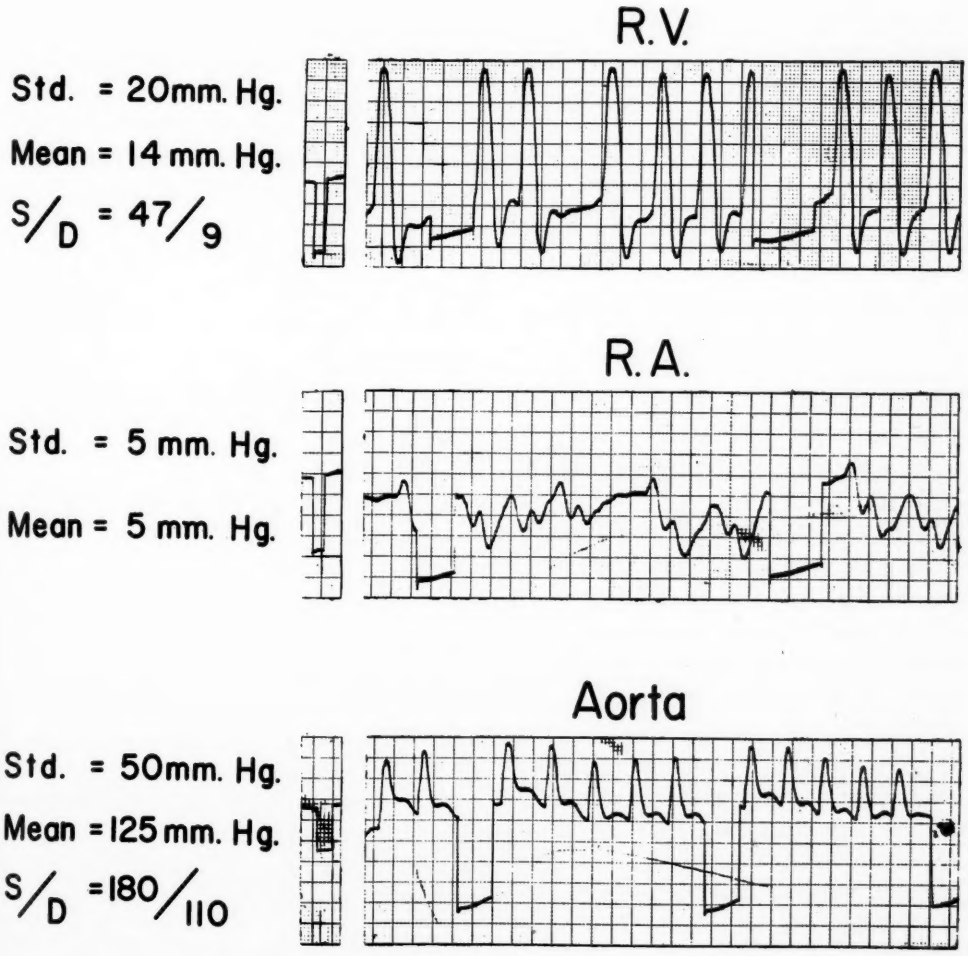

Fig. 3.—Sample pressure records from the right ventricle, right auricle, and abdominal aorta of an anesthetized dog, showing the calibration deflections on the left, and the pulse pressure records on the right, with average pressure recorded as the flat-bottom negative deflections obtained by switching the Record-Average switch to the Average position. (See text.)

DISCUSSION

The indication of average pressure will be absolutely correct under the following conditions:

1. The pressure wave is perfectly periodic, i.e., one pulse is exactly the same as the next.
2. The switching of the Record-Average switch takes place in zero time.
3. The electrocardiograph is perfectly linear and has no overshoot.

In regard to *1*, the character of the pulse is readily observed as the record is being written. Bearing in mind that the value given by the averaging circuit will be close to the correct average value of the last two or three beats prior to the throwing of the Average switch, the timing of the switching can be arranged to give nearly correct results even in the case of severe arrhythmia. In the event that the average pressure changes rapidly, the pressure tracing will indicate this by a displacement of the entire record upward or downward during the transition period. An average pressure should not be taken during a transition period.

In regard to *2*, the time constant RC in the averaging circuit is so large as compared with the switching time that this error will be negligible. It is easily calculated if the switching time is known.

Consideration *3* is the largest error in a typical case. The electrocardiograph will generally overshoot a few per cent. The performance of a particular electrocardiograph in this respect may be determined by depressing the 1 mv. standardizing switch contained within it. The overshoot per cent will be the same under these conditions as it is when the Average switch is thrown.

With reference to the average pressure deflections of Fig. 3, it will be observed that following the initial negative excursion, the stylus rises a certain distance and upon release of the Average switch, the pressure record is displaced upward this same amount for about two beats. The reason for this is explained by reference to Fig. 1. Following the switching from R to A, the capacitor discharges through the resistor, causing the voltage across the resistor to decay in an exponential fashion. The stylus records this decay as an upward slope. The extent of the upward deflection depends upon the length of time the switch is held in the average position. By the time the switch is returned to the R position, the voltage across C is less than that corresponding to average pressure. Since the capacitor voltage is in opposition to the average pressure voltage coming from the gauge, at the instant of switching to the record position the stylus deflects to a position higher than its correct position by an amount corresponding to the voltage lost by the capacitor while the switch was in the average position. This condition will correct itself in the few seconds required for the capacitor to charge again to the voltage corresponding to average pressure.

The pulse pressure and the pressure wave shape will be limited in accuracy only by the performance characteristics of the pressure gauge and the electrocardiograph. Since the requirements placed upon the electrocardiograph for pressure recording are much less stringent than those for recording electrocardiograms, the accuracy of the pressure recording will be better than the accuracy of the machine when used as an electrocardiograph.

SUMMARY

A dependable and portable apparatus for recording arterial and venous blood pressures on a direct-writing electrocardiograph has been described. The pressure records are of good accuracy, providing pulse contours, mean, systolic, and diastolic pressures. The limitations of the apparatus are discussed.

A SIMPLIFIED METHOD FOR THE PREPARATION OF ANTI-M AND ANTI-N TYPING SERA

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INTRODUCTION

THAT it is quite difficult to produce satisfactory anti-M and anti-N typing sera is a well-known fact. The production of these antisera is difficult for the following reasons: (1) since rabbits are immunized with type O human erythrocytes, either M or N, a high titer of heterologous hemagglutinins for human erythrocytes of groups A, B, and O usually results, and these antibodies must be absorbed from the serum; (2) in addition to the rise in homologous M or N titer, resulting from the immunization of the rabbits with OM or ON cells, a significant heterologous M or N antibody titer also occurs, probably due to the high degree of antigenic similarity between the M and N antigens, and this heterologous antibody must also be absorbed; (3) in the course of absorbing the heterologous hemagglutinins for the human A, B, and O cells, and the heterologous anti-M or anti-N agglutinin, a considerable amount of the homologous M or N antibody is absorbed as well. In addition to these technical difficulties, there is the problem of obtaining a panel of AM, BM, and OM human cells for the absorption of anti-N serum, or a panel of AN, BN, and ON cells for the absorption of anti-M serum. With the simplified method described in this paper, it becomes unnecessary to absorb the immune anti-M or anti-N serum with the complete heterologous cell panel mentioned previously; only one of the heterologous cell types is needed.

In a previous report,[2] we demonstrated the thermolability of the heterologous hemagglutinins for human erythrocytes in connection with the processing of antihuman globulin serum, produced in rabbits, and the thermostability of the antihuman-globulin antibody, in the same system. The technique consists of diluting the unabsorbed antihuman-globulin serum 1:10 with physiologic saline solution and heating this diluted serum in a water bath at 70° C. for sixty minutes. The heterologous hemagglutinins for human erythrocytes are destroyed, while the antihuman-globulin antibody remains stable and specific. Since the need for absorption of the heterologous hemagglutinins with human erythrocytes is eliminated, it was thought that this method could be utilized

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This investigation was supported (in part) by a research grant (A-77) from the National Institute of Health, Public Health Service, and a grant from the Leukemia Research Foundation, Inc., Chicago.

Received for publication, June 10, 1954.

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as a means of simplifying the production of anti-M and anti-N typing sera, provided the M and N antibodies could be demonstrated to be thermostable under the afore-mentioned conditions.

EXPERIMENTAL RESULTS

Preliminary tests with absorbed anti-M and anti-N typing sera demonstrated these antibodies to be thermostable at 70° C. for sixty minutes. Having ascertained the thermostability of the M and N antibodies at 70° C. for sixty minutes, the next step was that of demonstrating the practicality of this technique in the preparation of anti-M and anti-N serum of diagnostic value.

TABLE I. COMPARATIVE STUDY OF THE EFFECT OF HEATING AND ABSORPTION ON HETEROLOGOUS HEMAGGLUTININS IN AN ANTI-N SERUM

		a	b	c	d	e
TREATMENT OF SERUM		−H* −A‡	+H† −A	−H +A§	+H +A‖	+H +A¶
Cell Types	ON	128**	128	16	16	8
	OMN	128	64	8	8	4
	AM	128	128	2	0	0
	BM	64	32	4	1	0
	OM	64	64	4	0	0

*−H, serum not heated.
†+H, serum heated at 70° C. for sixty minutes.
‡−A, serum not absorbed.
§+A, unheated serum absorbed with AM type erythrocytes.
‖+A, first absorption of heated serum with AM type erythrocytes.
¶+A, second absorption of heated serum with AM type erythrocytes.
**Titer of serum which is the reciprocal of serum dilution showing a 1+ agglutination reaction.

Unabsorbed, undiluted anti-N serum, produced by the immunization of a rabbit with type ON human erythrocytes, was diluted 1:10 with physiologic saline solution. This unheated serum was then titrated against a panel of ON, OMN, AM, BM, and OM cells, according to the method of Davidsohn and Rosenfeld[1] (Table Ia); as an additional control, this diluted serum was heated at 70° C. for sixty minutes and titrated in parallel (Table Ib). The next step, according to the method of Davidsohn and Rosenfeld, is the absorption of the diluted serum with a mixture of AM, BM, and OM human erythrocytes, for the purpose of removing the heterologous hemagglutinins for the A, B, and O erythrocytes, and the heterologous M antibody. However, since the removal of these heterohemagglutinins was demonstrated to be practicable by the application of heat,[2] and since the N antibody was demonstrated to be thermostable, the following series of experiments were carried out in order to prove the effectiveness of this simplified technique: (1) in order to establish a base line, the 1:10 dilution of the unheated serum was absorbed with an equal volume of type AM human erythrocytes, only. According to the method of Davidsohn and Rosenfeld,[1] one would expect a drop in the M titer, when this partially absorbed serum is titrated against OMN, AM, BM, and OM cells, and some drop in the N titer, when titrated against the ON cells due to cross-absorption; however, the absorption of the heterologous hemagglutinins for the B and O human erythrocytes

does not occur unless the absorption is done with BM and OM erythrocytes (Table Ic); (2) then, since the method under test was demonstrated to destroy the heterologous hemagglutinins for the A, B, and O human erythrocytes by heating a sufficiently diluted serum at 70° C. for sixty minutes, this was performed on a sample; however, because the heterologous M antibody, which occurs simultaneously with the thermostable N antibody, is always present, one further step in the procedure is necessary. After heating the diluted serum, it is necessary to absorb with one type of M erythrocyte in order to remove this heterologous M antibody; this was accomplished by absorbing the diluted, heated serum with an equal volume of three times washed, packed human cells of the AM type. Table Id demonstrates the removal of the interfering heterologous hemagglutinins to the A, B, and O erythrocytes. The final trace of M antibody was removed by a subsequent absorption with .5 volume of the AM type cells (Table Ie).

Applying the same procedure with respect to the preparation of anti-M serum, a series of experiments were carried out. First of all, unabsorbed anti-M serum was diluted 1:10 with physiologic saline solution, treated in the following ways, and titrated against a panel of OM, OMN, AN, BN, and ON cells:

(1) Not heated, and not absorbed (Table IIa);

(2) Heated, but not absorbed (Table IIb);

(3) Not heated, but absorbed with ¾ volume of AN type cells only (Table IIc);

(4) Heated at 70° C. for 60 minutes, and absorbed with 0.75 volume of AN type cells only (Table IId);

(5) Second absorption of the 1:10 dilution with 0.5 volume AN type cells (Table IIe).

TABLE II. COMPARATIVE STUDY OF THE EFFECT OF HEATING AND ABSORPTION ON HETEROLOGOUS HEMAGGLUTININS IN AN ANTI-M SERUM

		a	b	c	d	e
	TREATMENT OF SERUM	−H* −A‡	+H† −A	−H +A§	+H +A‖	+H +A¶
Cell Types	OM	64**	64	16	8	8
	OMN	32	32	16	8	4
	AN	32	16	4	1	0
	BN	32	32	4	1	0
	ON	32	32	2	1	0

*−H, serum not heated.
†+H, serum heated at 70° C. for sixty minutes.
‡−A, serum not absorbed.
§+A, unheated serum absorbed with AN type erythrocytes.
‖+A, first absorption of heated serum with AN type erythrocytes.
¶+A, second absorption of heated serum with AN type erythrocytes.
**Titer of serum which is the reciprocal of serum dilution showing a 1+ agglutination reaction.

A comparison of the results in these tables demonstrates the applicability of this simplified method for the preparation of the anti-M, as well as the anti-N serum.

At the end of four months, stability and specificity tests on pilot lots of anti-M and anti-N sera prepared in this manner, and stored at room, refrigerator, and freezer temperatures, have demonstrated the sera to be satisfactory in all respects.

DISCUSSION

A simplified method for the preparation of specific anti-M and anti-N typing sera has been presented. The method has several significant advantages. First of all, since the heterologous hemagglutinins for the A, B, and O human erythrocytes are destroyed by heating, it becomes unnecessary to use more than one cell type in the absorption procedure, and in addition, preliminary inactivation of the serum at 56° C. for thirty minutes is not necessary. Second, since only one cell type is necessary for absorption of the serum, considerable time is saved in that less typing of cells has to be done, in connection with preliminary preparation for absorption; there is less washing of cells to do; and only one type of erythrocyte is needed instead of three. The one cell type is necessary in order to absorb the heterologous M or N antibody. Third, since the heterologous hemagglutinins for human A, B, and O erythrocytes are first destroyed by heating, it is quite probable that absorption of the heterologous M or N antibody is more effective. Finally, since the preliminary pilot testing of animals is usually a time-consuming task because of the necessity of absorption with the complete heterologous cell panel, this simplified method offers obvious time and labor saving advantages in all preparatory operations.

SUMMARY

A simple and reliable method for the production of anti-M and anti-N typing sera, of diagnostic value, is presented. The method is based on the principle that heating sufficiently diluted rabbit immune sera of the M and N types results in the destruction of the heterologous hemagglutinins for the A, B, and O human erythrocytes, without affecting the stability or specificity of the anti-M or anti-N antibody, since they are thermostable at 70° C for sixty minutes. Then, by absorption of this diluted, heated anti-M or anti-N serum with any one A, B, or O type erythrocyte of the heterologous M or N factor, a specific serum can be prepared with little difficulty.

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